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Modulation of NMDA receptor surface expression by DISC1 and its pathway partners

By

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Declaration

I can confirm that this thesis has been composed by Darragh Kevin Crummie, the work presented in this thesis is my own and that the work has not been submitted for any other degree of professional qualification except as specified.

Abstract

Disrupted in Schizophrenia 1 (DISC1) is a well supported risk factor for schizophrenia, bipolar disorder and major recurrent depression. DISC1 is a multifunctional multicompartmentalised scaffold protein with essential roles in neuronal proliferation, differentiation, migration and integration. DISC1 also modulates pathways of vital importance for neuronal signalling and plasticity. One of the major hypotheses for the cause of psychiatric illness is N-methyl-D-aspartate (NMDA) receptor hypofunction. It was observed that NMDA receptor antagonists can induce symptoms of schizophrenia in unaffected individuals, and exacerbate symptoms in patients with schizophrenia. Recent work in our laboratory showed that DISC1 complexes with NMDA receptors within the cell body and at synapse of neurons. Here I studied whether DISC1, or DISC1 missense variants, affect the trafficking of NMDA receptors. This was done by quantifying surface NMDA receptor expression in the presence of DISC1 or variant DISC1. I found that one common variant, 607F, causes a significant reduction in surface expressed NMDA receptors. I went on to show that DISC1 reduces the number of internalised receptors associating with early RAB5-containing endosomes. This indicates that DISC1 may be involved in the trafficking and recycling of NMDA receptors, a process that may be affected by the missense DISC1 variant 607F. Further to this I studied the effects on NMDA receptor trafficking of DISC1 pathway partners Nuclear Distribution Element 1 (NDE1) and Trafficking-protein kinesin binding 1 (TRAK1), both regulators of neuronal intracellular trafficking. Phosphorylation of NDE1 at T131 has been shown to be modulated by DISC1. Using phospho-mimic and phospho-dead NDE1 expression constructs I observed a significant reduction in the surface-expressed NMDA receptors in cells expressing the phospho-mimic form of NDE1. NDE1 may therefore be involved in the trafficking of NMDA receptors, and this role may be modulated by phosphorylation of NDE1. Finally, TRAK1 was shown to associate robustly with the GluN2B subunit, and to decrease the surface expression of NMDA receptors, most likely by sequestering them. The TRAK1-induced GluN2B sequestration may be an artefact, but the association of the trafficking molecule TRAK1 with this subunit may point towards a role in NMDA receptor trafficking. These proteins have been shown to associate with each other and may

form a complex in order to traffic NMDA receptors. Disruption of this complex by defective DISC1 expression may affect NMDA receptor trafficking. In the brain this could conceivably contribute to NMDA receptor hypofunction and the development of psychiatric illness.

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List of abbreviations

ADHD	Attention deficit hyperactivity disorder
AP1	Adaptor protein 1
AP5	2-amino-5-phosphonopentanoic acid
ATF4	Activating transcription factor 1
BBS4	Bardet-Biedl syndrome 4 protein
BrdU	bromodeoxyuridine
cAMP	cyclic adenosine monophosphate
Cdk1	cyclin-dependent kinase 1
CIT gene	citron rho interacting kinase
CNV	copy number variant
CO-IP	co-immunoprecipitation
COS7 cells	African green monkey kidney cell line
C-terminal	carboxy terminal
DB	dissection buffer
DISC1	Disrupted in schizophrenia 1
DISC2	Disrupted in schizophrenia 2
DIV	days in vitro
DLPFC	Dorsolateral pre-frontal cortex
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DPBS	Dulbecco's phosphate buffered saline
E.Coli	escherichia coli
EEA1	early endosome antigen 1
EGFR	epidermal growth factor receptor
ER	Endoplasmic reticulum
ERK1	extracellular signal-regulated kinase 1
FBS	foetal bovine serum
GABA	gamma-aminobutyric acid
GDP	guanine di-phosphate
GFP	Green flurescent protein
Grb2	growth factor receptor bound protein 2
GSK3β	glycogen synthase kinase 3
GTP	guanine tri-phosphate
GWAS	Genome wide association study
HEK-293	human embryonic kidney cells
IBMX	Isobutylmethylxanthine
IP	Immunoprecipitation
KD	Knock down
Kal-7	Kalerin-7
KIF	Kinesin family member
LAMP1	lysosomal-associated membrane protein 1
LAMP2	lysosome associated membrane protein 2

LB	Lysogeny broth
LIS1	Lissencephaly 1
LOD	Logerithem of odds ratio
LTD	long term depression
LTP	long term potentiation
MAGUK	membrane associated guanylate kinase
mEPSC	miniture post-synaptic currents
Miro	Mitochondrial Rho GTPase
MK-801	Dizocilpine NMDA receptor antagonist
NAT	Natural anti-sense transcript
NDE1	Nuclear distrabution protein-E 1
NDEL1	Nuclear distrabution protein-E like 1
NMDA	N-methyl D-aspartate
N-terminal	amine terminal
pA	pico-amp
PC12 cells	pheochromocytoma derived cell line
PCM1	pericentriolar material 1
PCP	phencyclidine
PDE4	Phospho-diesterase 4
PDZ	post synaptic density - drosphila disc large tumorsuppressor - zona occludens-1
pF	pico-farad
PFA	paraformaldehyde
PKA	protein kinase-A
PKC	protein kinase-C
PMT	Photomultiplier
PSD	post synaptic density
RAB11	Ras-related Protein 11
RAB5	Ras-related Protein 5
RAB7	Ras-related Protein 7
RAC1	Ras-related c3 botulinum toxin substrate 1
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interface
ROI	region of interest
rpm	revolutions per minute
S.O.C	super optimal broth
SAP102	synaptic associated protein
shRNA	Short hairpin ribonucleic acid
Su48	centrosome associated protein
TIM23	Translocase of the inner membrane
TM	Trans-membrane
TNIK	TRAF2 and NCK-interacting protein kinase
TRAK1	Trafficking and kinesin 1
TRAK2	Trafficking protein kinesin binding 2

TTX	tetrodotoxin
VLA2α	Very late antigen
WT	Wild type

1 Introduction

1.1 DISC1

1.1.1 t(1;11) family

The disrupted in schizophrenia 1 (DISC1) locus was originally identified in a large Scottish family with a high incidence of mental illness, which co-segregated with a t(1:11) translocation (St Clair et al., 1990). 87 members of the family have been karyotyped 37 of which carry the t(1;11)translocation (Blackwood et al., 2001). Detailed assessment was only possible in 29 of the family members with the translocation, and of these, 7 members had schizophrenia, 10 had recurrent major depression and 1 had bipolar disorder (Millar et al., 2001). Therefore 18 of 29 had a major mental illness whereas 0 of the 38 non-translocation carriers had a diagnosis of major mental illness. High logarithm of odds ratio (LOD) scores confirmed close linkage of the translocation to schizophrenia (LOD score 3.6), thus providing evidence for a causal link between the t(1:11) translocation and schizophrenia in this family. Furthermore when the diagnostic criteria were extended to include schizophrenia, bipolar disorder and recurrent major depression a highly significant LOD score of 7.1 was obtained, indicating that inheritance of the translocation contributes to susceptibility to developing a major mental illness (Blackwood et al., 2001).

The breakpoint regions on chromosome 1 and 11 were fine mapped and cloned, no evidence of a gene was found at the breakpoint on chromosome 11 (Devon et al., 1997, Millar et al., 1998) however two genes were discovered to be disrupted by the breakpoint on chromosome 1, DISC1 and DISC2 (Millar et al., 2001, Millar et al., 2000). Broadly speaking DISC1 has been found to be a multi-functional, multi-compartmentalised protein linked to pathways which regulate neuronal outgrowth (Millar et al., 2000) and intracellular trafficking (Atkin et al., 2010, Millar et al., 2001, Ogawa et al., 2013). Furthermore alterations in DISC1 levels affect neuronal precursor proliferation (Mao et al., 2009) neuronal migration (Young-Pearse et al., 2010, Kamiya et al., 2005, Duan et al., 2007) and neuronal integration (Duan et al., 2007). DISC1 therefore plays many important roles in brain development. DISC2 is thought to be a natural anti-sense transcript (NAT) of DISC1, which may negatively

regulate DISC1, however the function of DISC2 has yet to be determined (Chubb et al., 2007).

Subsequently a novel gene on chromosome 11 was found to be disrupted by the translocation named “Boymaw”. Two fusion proteins between Boymaw and DISC1 have been proposed by Zhou et al, one is a protein containing exons 1-8 of DISC1 fused to exons 4-6 of Boymaw (DB7) and the other is exons 1 and 2 from Boymaw fused to 9-13 of the DISC1 gene (DB13). DB7 when overexpressed in HEK 293 cells was found to be insoluble as western blot analysis showed their presence in the pellet, whereas the DB13 fusion proteins were found in the supernatant. To analyse the subcellular localisation of these fusion proteins rat hippocampal neurons were transfected with HA-tagged DB7 or DB13. DB7 appeared punctate in appearance whereas DB13 appeared diffuse throughout the cytoplasm, generation of these fusion transcripts could contribute to the pathogenesis of mental illness as the insoluble proteins could disrupt normal function of neuronal cells. Therefore the translocation event at t1:11 disrupts DISC1 and lead to the formation of fusion proteins which could in turn contribute to an increase in susceptibility of mental illness.

The wider significance of the DISC1 translocation in the cause of mental illness has been called into question. There has been a lack of GWAS data highlighting DISC1 as a risk factor for mental illness which some have interpreted to mean that DISC1 is not a risk factor for mental illness. An alternative view is that there are no common ancient variants in DISC1 and is therefore less likely to appear by GWAS, but there may well be population-specific variants which are detectable by smaller population-based family studies. For example family based studies have shown linkage between DISC1 and schizophrenia (Hennah et al., 2007) with additional loci which link to mental illness being identified once conditioned on the DISC1 risk haplotype e.g. nuclear distribution protein-E 1 (NDE1). Through a direct interaction DISC1 regulates NDE1 and is essential for several fundamental processes during brain development and its function is regulated by DISC1 (Bradshaw et al., 2009)

thereby providing evidence of a shared risk pathway disruption of which leads to an increase in susceptibility in schizophrenia.

1.1.2 Protein structure

The DISC1 gene consists of 13 exons which encodes a protein of 854 amino acids. The human DISC1 gene produces a number of alternatively spliced transcripts of which there are four major transcripts; Long, exons 1-13; Long variant, this transcript excludes 66 amino acids from the distal end of exon 11; Short, this transcript has an alternative 3'-UTR in exon 9; and Extremely short, which has an alternatively spliced exon 1a causing termination of transcription two intronic codons after exon 3. (Millar et al., 2001, Taylor et al., 2003). In the human brain DISC1 is predominantly expressed as the long transcript with low levels of the alternatively spliced DISC1 transcripts being expressed (Lipska et al., 2006). Due to the fusion of chromosomes 1 and 11 after the translocation event a number of fusion transcripts with protein coding potential have been found. A study into the potential function of these fusion transcripts showed the exogenous expression can induce abnormal mitochondrial morphology and membrane potential (Eykelenboom et al., 2012). The fusion transcripts also formed large stable protein aggregates which could be linked to those observed post mortem in patients with mental illness (Leliveld et al., 2008, Korth, 2009). However, these transcripts are very scarcely detected in patient lymphoblastoid cell lines and therefore any data should be interpreted with caution.

There is no experimentally determined 3D structure for DISC1 and therefore the field has relied on bioinformatic predictions and biophysical studies (using fragments of DISC1) to gain information about the structure of DISC1. The structure of DISC1 is predicted to form a largely unstructured N-terminus (~1-350aa) with the C-terminus adopting a helical structure with the potential to form a coiled-coil (~350-854aa) (Millar et al., 2001, Millar et al., 2000, Soares et al., 2011).

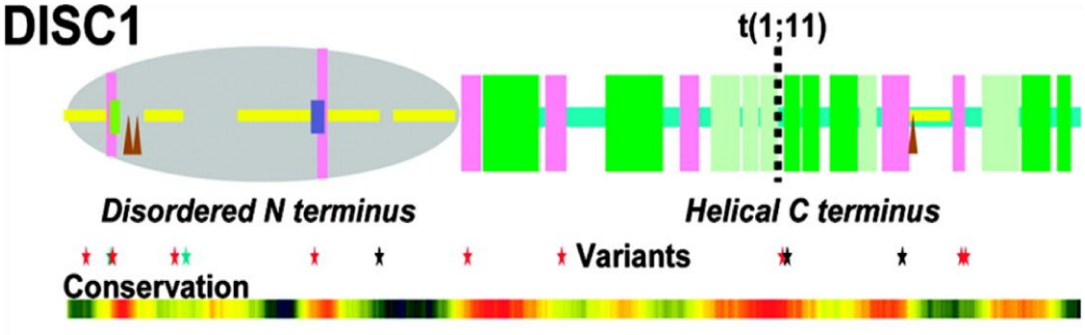

Figure 1.1 Predicted structure of DISC1 – adapted from Soraes et al 2011

Predicted DISC1 structure grey oval represents the disordered N-terminal head domain with the yellow lines representing regions of disorder. The green and pink boxes represent the helical nature of the C-terminal of DISC1 with the chromosomal translocation breakpoint shown in black. Stars represent the location of different variants. Also a heat map showing the conservation of regions of DISC1 with Red being highly conserved and blue not conserved.

It has been recently shown that DISC1 can assemble into octomers using a self-association domain located within the C-terminus of DISC1 to form dimers. These dimers are then used to form octomers. The authors of this study proposed a model where the octomers of DISC1 interact with nuclear distribution protein-E like (NDEL1) to create DISC1/NDEL1 complexes (discussed in 1.1.5). In addition a common variant identified as a risk factor for schizophrenia S704C-DISC1 has been shown to affect the oligomeric status of DISC1 (discussed in 1.1.5). Previous studies reported 704C-DISC1 having increased NDEL1 binding when compared to WT-DISC1. The authors of this study determined that the NDEL1 binding site was located in the C-terminus of DISC1 and predicted the 704C variant caused the formation of higher order oligomers rather than increasing the binding affinity to NDEL1 (Leliveld et al., 2008). Further to this Leliveld et al characterised the multimerisation of DISC1 fragment 598-854 and found it existed as dimeric, octameric and miltimeric species (Leliveld et al., 2008). Under physiologically relevant conditions the authors found the fragment reduces aggregation but only the octomeric species interacted with NDEL1, indicating that oligomerisation was necessary for interaction between the two proteins. Finally the authors found NDEL1 did not bind aggregates of FL-DISC1 (Leliveld et al., 2008). Whilst bioinformatic predictions have been useful in

determining a general structure, without an experimentally determined structural model for DISC1 there are limits to what can be determined about structure-function relationships with other proteins and how single nucleotide polymorphisms (SNP) may affect the structural integrity of DISC1 (Soares et al., 2011)

1.1.3 Expression in tissue

DISC1 has been found to be expressed in a number of different locations in human adult and foetal tissue at both the protein and RNA level, which include the brain, the heart and the placenta (Millar et al., 2000, James et al., 2004). Within the human brain DISC1 is expressed in many brain regions with its highest expression within the dentate gyrus of the hippocampus (James et al., 2004, Lipska et al., 2006). This was also observed in mouse brain samples where the highest DISC1 expression was found in the dentate gyrus and lower expression in the cerebellum, cerebral cortex, ammons horn and the olfactory bulbs (Ma et al., 2002). In mice DISC1 is expressed throughout the brains development, but there are expression peaks at both embryonic day 18 (E18 (active neurogenesis)) and P35 (onset of puberty) (Brandon et al., 2004).The studies cited here were carried out using in situ hybridisation of RNA and therefore the findings are quite robust, with the exception of James et al where the authors used several DISC1 antibodies to determine localisation of DISC1 (James et al., 2004) (and therefore the findings are quite robust) and Schurov et al where the authors only used one DISC1 antibody and therefore the results should be interpreted with caution.

At the subcellular level DISC1 has a broad expression pattern where it is located within multiple cellular compartments (Ozeki et al., 2003, James et al., 2004, Kamiya et al., 2005, Morris et al., 2003, Ogawa et al., 2005, Kamiya et al., 2006, Miyoshi et al., 2003, Brandon et al., 2004, Brandon et al., 2005, Miyoshi et al., 2004, Shinoda et al., 2007, Taya et al., 2007). Endogenous DISC1 has been shown to co-localise with markers for the mitochondria (Ozeki et al., 2003, Millar et al., 2000, Millar et al., 2005b, Brandon et al., 2005), the golgi apparatus (Kuroda et al., 2011, Lepagnol-Bestel et al., 2013) and the centrosome (Kamiya et al., 2006, Kamiya et al., 2005) as

well as markers for the cytoskeleton (Brandon et al., 2004, Brandon et al., 2005, Atkin et al., 2012) and the nucleus (Millar et al., 2005a). In neurons DISC1 is also been shown to localise to a number of different subcellular locations including post-synaptic density (PSD), the cell body, neurites and the nucleus (Taya et al., 2007, Shinoda et al., 2007, James et al., 2004, Ozeki et al., 2003, Ogawa et al., 2005, Brandon et al., 2005, Miyoshi et al., 2003, Bradshaw et al., 2008). Furthermore DISC1 has been implicated in membrane targeting and vesicle tethering via its interaction with dynein and the exocyst complexes (Mead et al., 2010) therefore opening up a potential role of DISC1 in neuronal signalling (discussed in 1.1.4).

One study used electron microscopy of human samples to visualise DISC1 expression (Kirkpatrick et al., 2006). Kirkpatrick et al discovered DISC1 expression within axon terminals, the PSD and dendritic spines. Furthermore the authors showed an association between DISC1 with the cell body, with ribosomes within dendritic shafts and with the endoplasmic reticulum (ER) (Kirkpatrick et al., 2006). These studies have determined that DISC1 is widely expressed throughout the cell and throughout neurons which, coupled with the many known interactors of DISC1, indicate DISC1 may have (and be essential for) multiple functions within the cell.

Although DISC1 has been shown to localise to many subcellular compartments these studies must be interpreted with caution. This is due to a large number of different DISC1 antibodies being used by different groups (15-33) many of which are made “in-house” by the labs and the data has not been replicated by independent groups. This has lead to a debate over suitable antibodies which can detect full length DISC1. It was found that 129s6/SvEv carry a 25bp deletion in exon 6 of DISC1 which leads to a premature stop codon in exon 7 (Clapcote and Roder, 2006) The predicted protein which would be produced because of this stop codon was not detected, indicating that it may form an unstable protein which is degraded and therefore no full-length DISC1 is detected in this mouse. A study in 2007 compared antibody detection of full length DISC1 using antibodies that were generated from eight independent groups and generated against more than 10 different epitopes

within DISC1. The authors found that all the antibodies they tested detected full length DISC1 at similar levels in 129s6/SvEv strain of mice with the exception of one antibody generated by Dr J. A. Gogos. The 129s6/SvEv mouse does not express full-length DISC1 and therefore with the exception of the Gogos antibody the antibodies tested may not detect DISC1.

Since then it has been suggested that a consensus was agreed that studies of DISC1 should use two different DISC1 antibodies and yield consistent western blot and IP results. Many of the studies referenced here were published before this consensus but the localisation of DISC1 to some subcellular compartments has been confirmed by multiple groups using overexpressed protein as well as endogenous protein and therefore there is strong evidence for DISC1 expression at the; centrosome (Morris et al., 2003, Miyoshi et al., 2004, Bradshaw et al., 2008, Kamiya et al., 2008), PSD (Kirkpatrick et al., 2006), mitochondria (Ramsey et al., 2011, Paspalas et al., 2012, Ogawa et al., 2013), cytoskeleton (James et al., 2004, Miyoshi et al., 2003, Brandon et al., 2004, Brandon et al., 2005, Atkin et al., 2012), nucleus (Sawamura et al., 2008, Malavasi et al., 2013) and golgi (Kuroda et al., 2011, Lepagnol-Bestel et al., 2013). DISC1 localisations which have been suggested but need more evidence are the ER and ribosomes, (Kirkpatrick et al., 2006).

1.1.4 DISC1 function

1.1.4.1 DISC1 in brain development

DISC1 has been described as a multifunctional, multicompartmentalised “hub” protein due to its many interactors and functions. A yeast 2-hybrid screen using DISC1, and DISC1 fragments, as bait revealed over 200 potential interactors, many of which have now been confirmed biochemically in neuronal or mammalian cell lines (Morris et al., 2003, Millar et al., 2003, Ozeki et al., 2003, Camargo et al., 2006). Additional yeast 2-hybrid studies found DISC1 interactors linking DISC1 to the centrosome, neurotransmission and neuronal plasticity (Hattori et al., 1994, Miyoshi et al., 2004). Disruption of the DISC1 gene in the t(1;11) family could not only disrupt the formation and function of DISC1 but also a number of interacting

proteins leading to the disruption of signalling pathways and intracellular processes (Millar et al., 2003, Hennah et al., 2007).

The hippocampus is a major site of neuronal precursor proliferation whereby new neurons are constantly being created throughout the life of the mammalian brain. DISC1 has been found to play a critical role in this process (Mao et al., 2009). Knockdown of DISC1 using electroporation of shDISC1 at E13, caused a significant reduction in cells within the sub and ventricular zone of the hippocampus (Mao et al., 2009). Whereas, the overexpression of full length DISC1 caused a significant increase in proliferation of newborn cells within sub ventricular and ventricular zones. Further to this the same authors showed in adult brain knockdown of DISC1 also caused a significant reduction in the proliferation of newborn cells (Mao et al., 2009).

Several studies have shown that the knockdown of DISC1 in the developing mouse brain can cause deficits in neuronal migration. One study showed knockdown of DISC1 *in utero* inhibits the migration of neurons from the ventricular zone to the cortex. The authors electroporated embryos at E14.5 with RNAi to knockdown DISC1 and analysed the brains at postnatal day 2 (P2) where they observed a significant reduction in neuronal migration as there were few labelled neurons within the upper layers of the cortex, but still detected neurons within the ventricular zone. Additionally the authors noticed (in the neurons which had migrated) abnormalities in the branching structure at P14 when branching and the formation of circuits is usually quite extensive (Kamiya et al., 2005). Conversely DISC1 knockdown in adult mice leads to overextended migration of new-born neurons in the hippocampus and neurons which had DISC1 expression knocked down exhibited a much more complex branching structure indicating DISC1 may be involved in dendritic branching development in new-born adult neurons (Duan et al., 2007). Although these are contrasting findings Duan et al offered a potential explanation into why this may be as the authors speculated that the differences

between these studies may suggest that DISC1 regulates the detection of cues that aid the migration of neurons in the developing and adult brain (Duan et al., 2007).

DISC1 also plays a critical role in neuronal integration (Duan et al., 2007, Faulkner et al., 2008, Kvajo et al., 2008). Knockdown of DISC1 in the adult dentate gyrus leads to the accelerated formation of functional GABAergic and glutamergic synaptic inputs to new neurons, as increased numbers of dendritic spines were observed in new neurons after 2 weeks post knockdown of DISC1 whereas in control cells an increase in the number of dendritic spines was only observed after 4 weeks (Duan et al., 2007). Further evidence for DISC1s role in neuronal integration was provided by Fulkner et al who showed DISC1 knockdown in the dentate gyrus of adult mice lead to an increase in axonal growth of newborn neurons which projected outwith the CA3 region of the hippocampus (Faulkner et al., 2008). In control cells axons did not grow beyond the CA3 region indicating DISC1 regulates the correct integration of newborn neurons (Faulkner et al., 2008). DISC1 therefore plays a critical role in the integration of newborn neurons into the dentate gyrus of adult mice.

DISC1 is present at the synapse where it interacts with the synaptic protein PSD95. Additional studies have showed exogenous expression of DISC1 localises to dendritic spines (Bradshaw et al., 2008, Hayashi-Takagi et al., 2010). Functional studies have yet to be performed therefore the exact role of DISC1 at the synapse has yet to be elucidated. However one study has determined that DISC1 modulates size and density of dendritic spines via an interaction with Kal-7. Kal-7 is a GTP/GDP exchange factor for RAC1 and regulates spine morphology and plasticity. DISC1 anchors KAL-7 in a protein complex and controls access of Kal-7 to RAC1 thereby regulating dendritic spine size and density (Hayashi-Takagi et al., 2010). Furthermore DISC1 knockdown results in a decrease mobility of synaptic vesicles and an accumulation of vesicles along neuronal processes indicating DISC1 has a role in vesicle trafficking within neurons (Flores Iii et al., 2011). Pre-synaptically DISC1 may play a role in the regulation of glutamate release since the targeted

expression of a C-terminal truncated DISC1 disrupts glutamate vesicle fusion at the synapse (Maher and LoTurco, 2012).

Therefore DISC1 plays a critical role in the development and DISC1 can affect a number of different processes within the brain; neuronal migration, integration, neuronal precursor proliferation and neuronal signalling. Defective expression of DISC1 or any of its interactors that are also involved in these processes are likely to have a detrimental effect on brain development and function.

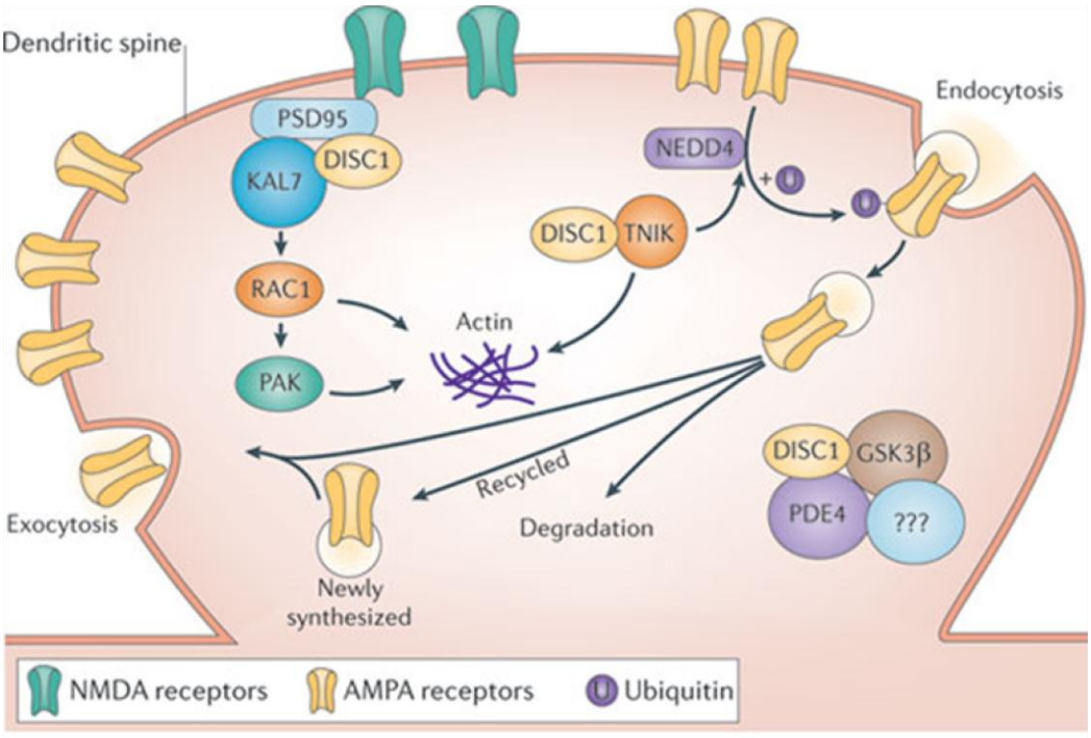

Figure 1.2 Potential DISC1 function at the synapse adapted from Brandon and Sawa 2011

This figure shows DISC1 potential interaction with AMPA receptors via TNIK where DISC1 via TNIK photphorylates AMPA receptors which are then endocytosed and either recycled or degraded. DISC1 may also have function with NMDA receptors via PSD95. DISC1/PDE4/GSK3β complex may provide additional function via phosphorylation.

1.1.4.2 DISC1 in intracellular trafficking

Within the cell DISC1 has a number of functions, that could influence its role in neurogenesis and neurosignalling, but this introduction will specifically examine the role of DISC1 in intracellular trafficking. Other DISC1 functions at the subcellular

level have been reviewed previously (Chubb et al., 2007, Thomson et al., Soares et al., 2011). Knockdown of DISC1 causes inhibition of axonal transport of various cargoes, which suggests a general role in microtubule based intracellular trafficking. More specifically DISC1 has been shown to interact with the microtubule motor proteins kinesin (Taya et al., 2007) and dynein (Kamiya et al., 2005) and interacts with a number of known trafficking molecules; LIS1, NDEL1, NDE1, 14-3-3α and TRAK1 (Ogawa et al., 2005, Ozeki et al., 2003, Morris et al., 2003, Brandon et al., 2004).

Dynein is a minus-end retrograde motor protein which has three main functions within the cell; to carry various cargoes away from the cell periphery (Roberts et al., 2013); assist in assembling microtubules into the spindle during cell division (Heald et al., 1996, Merdes et al., 1996); and exert tension on microtubules in order to pull the cytoskeleton in various directions. For example in migrating neurons, to aid movement dynein pulls the cytoskeleton towards the leading edge of the neuron (Tsai et al., 2007). Cytoplasmic dynein interacts with the proteins NDE1/NDEL1, LIS1 and dynactin, which are key regulators of its function (Roberts et al., 2013). Endogenous DISC1 was shown to coimmuniprecipicate with dynein intermediate chain in PC12 cells (Kamiya et al., 2005). Furthermore knockdown of DISC1 using RNAi in PC12 cells caused a reduction in neurite outgrowth an effect which was rescued by overexpression of WT-DSIC1. Expression of a C-terminally truncated form of DISC1 consisting of amino-acids 1-597 which mimics the translocation, caused the displacement of DISC1, LIS1 and dynactin from the centrosome and ultimately inhibits the anchoring of dynein to the centrosome leading to a disorganised microtubule network and a reduction in neurite outgrowth (Kamiya et al., 2005). Kamiya et al suggested that the failure of DISC1 mediated recruitment of the motor complex to the centrosome underlies the reduced neurite outgrowth and cortical migration deficits observed in neurons after DISC1 knockdown or overexpression of 1-597 (Kamiya et al., 2008). This could be due to a reduction of nucleokinesis (movement of the nucleus after the extension of the leading edge of the cell during cell migration) for which both the DISC1 interactors dynein and

kinesin are essential. Therefore a reduction in DISC1 levels would be predicted to result in a reduction in neuronal migration and outgrowth. DISC1 therefore associates with dynein and is critical for dynein function in relation to neuronal outgrowth and microtubule organisation and therefore is well placed to also play a role in dynein mediated intracellular transport, however this has yet to be studied.

Kinesin is a plus end molecular motor, which moves various cargoes such as synaptic vesicle precursors, mitochondria, NMDA receptor vesicles, AMPA receptor vesicles and mRNAs. In most cases kinesin motors recognise and bind their cargoes through scaffold proteins or adaptor protein complexes (Hirokawa et al., 2010). DISC1 interacts with kinesin-1 and can therefore potentially link several proteins and protein complexes to kinesin, and regulate their movement and localisation within the cell. DISC1 links the LIS1/NDE1/14-3-3ε complex with kinesin and regulates the transport of the complex in neurons, which leads to axon elongation (Taya et al., 2007). Further to this, DISC1 can regulate the transport of the growth factor receptor bound protein 2 (Grb2) (Shinoda et al., 2007). Grb2 links cell surface receptors to intracellular signalling pathways (Lowenstein et al., 1992) and forms a ternary complex with DISC1 and kinesin-1. In growth cones of rat hippocampi DISC1 and Grb2 are associated with the microtubules (Shinoda et al., 2007). Furthermore knockdown of kinesin caused a reduction in Grb2 motility and accumulation in growth cones similarly DISC1 KD also leads to an accumulation of GRB2 in growth cones and therefore they may be related in function (DISC1 and Kinesin), thus DISC1 is required for the kinesin-dependent movement of Grb2 along microtubules (Shinoda et al., 2007).

DISC1 expression levels have been shown to influence the number of motile mitochondria within neurons (Atkin et al., 2010) and DISC1 has been found to promote anterograde axonal mitochondrial trafficking within neurons (Ogawa et al., 2013). However the latter study failed to replicate the influence of DISC1 upon the number of motile mitochondria, therefore additional work is required to further elucidate the precise function of DISC1 in mitochondrial trafficking. However recent

studies showed DISC1 also associates with TRAK1 and Miro1 which link mitochondria to kinesin (Ogawa et al., 2013, Brickley et al., 2005, MacAskill et al., 2009), And therefore it is likely, through a direct interaction with TRAK1, that DISC1 influences mitochondrial trafficking within neurons.

Through Yeast 2-hybrid studies an interaction between DISC1 and EXOC1 (a component of the exocyst complex, which is essential for targeting exocytic vesicles to specific docking sites on the plasma membrane) has been found (Camargo et al., 2006). Mead et al further characterised the interaction by showing co-immunoprecipitation of endogenous DISC1 and exogenous EXOC3 in HEK-293 cells (Mead et al., 2010). DISC1 was also shown to be critical in vesicle transport in neurons as DISC1 knockdown resulted in a reduction of vesicle movement in neurons leading to an accumulation of vesicles along processes (Mead et al., 2010). This indicates DISC1 plays a central role in vesicle movement in neurons (Flores Iii et al., 2011).

The literature shows that DISC1 can act as a scaffold to link various proteins to dynein and kinesin motors to regulate their transport and localisation within the cell. DISC1 therefore plays an essential role in intracellular trafficking but due to its large number of protein interactors its full role in trafficking is still to be determined.

Within the developing brain disruption to DISC1 function or expression could lead to a reduction in; axonal elongation via its interaction with LIS1/NDE1/14-3-3ε and kinesin, a reduction in anterograde cargo movement via its interaction with kinesin which could lead to deficits in neuronal signalling and synaptic activity. Via DISC1s interaction with dynein, neuronal migration and intracellular trafficking would be affected which could lead to brain development and removal of synaptic protein. Therefore DISC1 likely plays an essential role in brain development and neuronal transport with further complexity still to be discovered.

1.1.5 DISC1 variants and mutants

Within the DISC1 gene three common amino acid variants have been identified which associate with mental illness: S704C, L607F and R264Q (reviewed in (Chubb et al., 2007, Bradshaw and Porteous, 2012)). Studies of R264Q have R264Q has been shown to affect cortical thickness within the lateral occipital gyrus (Brauns et al., 2011). DISC1 has been shown to regulate the activity of GSK3β through direct interaction, thereby regulating Wnt signalling (Mao et al., 2009). The same authors showed in a later study that the DISC1-264Q cannot rescue the decrease in neuronal progenitor proliferation observed after DISC1 knockdown (electroporation of shRNA and either DISC1 or DISC1-264Q was carried out at E13 and analysis was at E16). Finally the authors showed that DISC1-264Q also significantly reduced Wnt signalling in human lymphoblast cells (Singh et al., 2011). There is also a growing body of biological evidence for causative effects of the S704C and L607F variants.

There is genetic evidence linking S704C variant to mental illness. Callicot et al found evidence for variation at 704C in one of 3 independent cohorts and co-segragation of schizophrenia (Callicott et al., 2005). Subsequently other groups also observed a co-seragation with 704C and mental illness in Han-Chineese populations (Qu et al., 2007) and in Japanese populations a co-segragation of 704C and major depression (Hashimoto et al., 2006). Interestingly the 704C allele also co-segregated with cognitive defects in both healthy and patients with schizophrenia and 704C allele carriers performed worse in cognitive flexibility tests (Wisconsin card sorting test)(Di Giorgio et al., 2008).

Variation at position 704 was identified as a risk factor for mental illness by Ekelund et al (Ekelund et al., 2004). Since then this polymorphism has been associated with altered grey matter volume in the hippocampus and prefrontal cortex (Callicott et al., 2005, Cannon et al., 2005). Functional imaging studies confirmed that the polymorphism S704C lead to functional differences within the brain. First of all although patients performed similarly during testing, S704 homozygotes showed an increase in hippocampal-DLPFC functional coupling during memory encoding which indicates higher activity in this region (Di Giorgio et al., 2008). Additional studies

also showed differences between S704 homozygotes and patients with schizophrenia or bipolar disorder in the synaptic activity within a number of regions within the brain (Prata et al., 2008) therefore providing further evidence for a causative effect of this polymorphism.

At the molecular level the C704 variant is associated with reduced activity of the kinases extracellular signal-regulated kinase 1 (ERK1) and RAC-alpha serine/threonine-protein kinase (AKT) when compared to S704 (Hashimoto et al., 2006). The ERK signalling pathway controls a number of processes within the cell including proliferation and motility (Kolch, 2005), therefore disruptions to the homeostatis of this signalling pathway could be detrimental the health of the cell. Analysis of brain tissue from C704 carriers showed a trend towards reduced PCM1 immunoreactivity in glial cells when compared to S704 homozygotes (Eastwood et al., 2010). PCM1 is essential for microtubule assembly, and DISC1 (along with Bardet-Biedl Syndrome 4 protein (BBS4), which is localised to the centrosome and contributes to the maintenance of microtubular dynamics, as well as intracellular transport and ciliary function (Kamiya et al., 2008), recruit PCM1 to the centrosome. Disruption of this process leads to neuronal migration deficits during cortical development (Kamiya et al., 2008), therefore DISC1-704C carriers may suffer from neuronal migration deficits. Finally the S704C polymorphism was found to have an effect on NDEL1 binding, with the 704C-DISC1 having slightly increased affinity for NDEL1. As discussed in 1.1.2 DISC1-704C oligomerises more extensively than DISC1-704S, and DISC1 octomers associate with NDEL1 therefore the 704C vaiant has a stronger association with NDEL1 (Leliveld et al., 2008). The 704C variant is more strongly associated with abnormal behavioural phenotypes(Leliveld et al., 2009). It is, therefore, possible that aberrant oligomerisation of DISC1 could affect behaviour, however more work is needed to draw any firm conclusions (Leliveld et al., 2009).

Another common variant is L607F (Cannon et al., 2005, Hodgkinson et al., 2004). Variation in DISC1 at 607 was first identified as potential risk factor in mental illness

by Hodgkinson et al (Hodgkinson et al., 2004). Using a case-control data set of individuals the authors found an undertransmission of a common haplotype and evidence of association of schizophrenia, schizo affective disorder and bipolar disorder with multiple haplotypes which included a strong association of 607F carriers and schizoaffective disorder (Hodgkinson et al., 2004). A second group looked at family trios in French and Algerian populations where they found the L-allele of L607F was over transmitted in both populations and assessing for schizophrenia the authors found patients with the F scored significantly higher on the scale for the assessment of negative symptoms (which is a scaling system used to determine the severity of negative symptoms of mental illness), thus providing further evidence for a lack of 607F and increasing influence of schizophrenia (Lepagnol-Bestel et al., 2013). Callicot used a family based association and looked at the functional impact of DISC1 variation on phenotypes linked to hippocampal formulation but only found evidence for an effect with the 704C risk allele (Callicott et al., 2005).

Functionally variation at position 607 has been associated with a greater severity of the positive symptoms of schizophrenia and volume reduction in the superiorfrontal, anterior cingulate gyri (Szeszko et al., 2008). Similar to the findings with S704C, this variant influences cortical thickness and DLPFC activation, with the Phe variant having a detrimental effect when compared to Leu homozygotes (Brauns et al., 2011). However the biological mechanisms underlying these traits have yet to be determined.

Within the cell DISC1-607F is associated with reduced PCM1 immunoreactivity and reduced noradrenaline release (Eastwood et al., 2009). This could be due to the location of the polymorphism, which is located within a predicted leucine zipper, changes in this region have been shown (in other models) to disrupt stability, oligomeric state and orientation of the coiled-coil helices, and the change from Leu to Phe would be predicted to disrupt coiled-coil formation (Soares et al., 2011). DISC1-607F has also been shown to be influence mitochondrial trafficking, with

DISC1 carrying the Phe allele unable to rescue mitochondrial trafficking deficits induced by DISC1 knockdown (Atkin et al., 2010). Finally , the Phe variant impairs nuclear targeting of DISC1, which leads to a decreased ability of DISC1 to regulate ATF4-dependent transcription (Malavasi et al., 2013). DISC1 interacts with the stress response factor ATF4, which has a number of functions within the cell including activation of gene transcription (Ameri and Harris, 2008), regulation of synaptic plasticity (Green et al., 2008, Costa-Mattioli et al., 2007) and promote cell survival or cell death pathways (Fawcett et al., 1999, Ohoka et al., 2005). Through decreased association the 607F carriers may have altered ATF4 signalling which could lead to abnormalities in synaptic communication.

Rare amino acid sequence variants have also been identified within DISC1. R37W is an ultra-rare polymorphism which was first identified in a large study performed by Song et al (Song et al., 2008). The group originally identified 5 novel mutations; G14A, R37W, S90L, R418H and T603I, in 6 patients out of 288 diagnosed with schizophrenia but not in 288 matched controls or a subsequent sample of 5000 unaffected individuals (Song et al., 2008). Additionally this variant was identified in a single individual diagnosed with depression in a separate study of 653 patients with a major mental illness (schizophrenia, bipolar disorder or major depressive disorder) and 889 matched controls (Thomson et al., 2013b). Within this family there were two carriers of the R37W mutation, one of whom has been diagnosed with major depressive disorder and the other with anxiety (Song et al., 2008, Thomson et al., 2013b) and another member of the family who did not carry the mutation but has been diagnosed with bipolar disorder.

To date there have been few studies of the effect of 37W at the subcellular level. One recent study demonstrated that 37W alters the mitochondrial localisation of DISC1 from punctate to tubular, while blocking DISC1's ability to promote anterograde mitochondrial movement (Ogawa et al., 2013). This study also showed that 37W increases DISC1/TRAK1 association in COS7 cells (Ogawa et al., 2013). Furthermore DISC1-37W overexpression in COS7 cells results in decreased nuclear

abundance and, similar to the variant form of DISC1 carrying 607F, has a decreased ability to inhibit ATF4 mediated transcription (Malavasi et al., 2013).

The R37W polymorphism is located within a tetra-Arginine stretch that is a known nuclear localisation sequence (Sawamura et al., 2008). Therefore disruptions to this region may affect the nuclear targeting of DISC1 and thereby may explain the reduction in nuclear abundance observed for DISC1-37W (Malavasi et al., 2013). Furthermore it has been hypothesised that the 37W alteration could affect the positive charge created by the tetra-arginine region and thereby affecting formation of ionic bonds with other proteins (Ogawa et al., 2013). In addition, the authors speculated that the presence of an aromatic ring introduced by the tryptophan may alter the conformation of the protein in this region making binding more favourable in the case of TRAK1 (Ogawa et al., 2013).

Taken together studies have shown that DISC1 sequence variants can dramatically alter its function, leading to deficits in several processes. Mitochondrial trafficking is one of the processes now know to be affected by at least two of the variants, 37W and 607F. While these studies have focused upon mitochondrial trafficking, DISC1 is also known to modulate synaptic vesicle transport (Flores Iii et al., 2011). Moreover DISC1 interactors also regulate motility of other cargoes e.g. in addition to its involvement in mitochondrial transport, TRAK1 also directs endosome to lysosome trafficking, and modulates $GABA_A$ receptor trafficking. There is potential, therefore, for DISC1 to have a general role in the regulation of intracellular trafficking.

1.2 The NMDA receptor

1.2.1 NMDA receptors in psychiatric illness

NMDA receptor hypofunction has long been implicated in psychosis. Since the 1970's, post mortem studies of patients with schizophrenia have shown abnormal glutamate receptor binding levels and abnormal glutamate levels in the cerebrospinal fluid in patients with schizophrenia. More recently it has been shown that the NMDA receptor antagonists PCP and ketamine can model both the positive and negative symptoms of the disease in unaffected individuals (Moghaddam and Krystal, 2012, Krystal et al., 1994). What's more these drugs exacerbate the symptoms in patients with schizophrenia indicating a link between NMDA receptor hypofunction and schizophrenia (Malhotra et al., 1997, Krystal et al., 1994). Furthermore patients with schizophrenia can present with hypofrontality where they show decreased activity in the prefrontal cortex, further implicating hypofunction of NMDA receptors. Although there is a strong case for NMDA receptor hypofunction contributing to mental illness, the mechanisms of how this occurs have yet to be elucidated. There has been extensive testing for variation in the genes encoding receptor subunits and an association with mental illness, with no significant data being published. Using independent cohorts a weak association in some genes has been found but any genetic evidence linking NMDA receptors or other receptor subunits ($GABA_A$, AMPA, kinate ect) has been very limited (O'Donovan and Owen, 1999).

There is some genetic evidence linking the NDMA receptor to schizophrenia via NRG1. NRG1 is a pleiotropic growth factor which has a multitude of different roles including but not limited to synaptogenesis, neurotransmission and neuronal migration. NRG1 can act via the ErbB family of transmembrane tryrosine kinases where it activates a signalling cascade which can modulate a number of receptors (NMDA, Dopamine, and $GABA_A$, for a detailed review see Deng et al 13 (Deng et al., 2013)). Several linkage studies have shown chromosome 8p to link to schizophrenia (for detailed review see Harrison and Law (Harrison and Law, 2006)). Therefore in part linking NMDA receptor signalling to mental illness however this is a tentative

link as NRG1 acts on several different signalling pathways. As DISC1 is a known risk factor for schizophrenia and potentially binds to NMDA receptors (peptide S. Mackie and Murdoch unpublished) this further implicates the NMDA receptor in mental illness

The NMDA receptor is one of the major excitatory receptors in the mammalian brain. The NMDA receptor is an ionotropic coincidence detector (as it is regulated simultaneously by voltage and two ligands, glutamate and glycine) and is part of the glutamate receptor family which also encompasses AMPA and Kainate receptors. Whereas AMPA receptors are the primary mediators of fast excitatory transmission under basal signalling conditions, NMDA receptors are responsible for slow excitatory transmission and are highly permeable to Ca^{2+} ions. The influx of Ca^{2+} into the cell activates signal transduction cascades, which in turn regulate a number of cellular processes including synaptogenesis, synaptic modification, elimination and long term potentiation (LTP), a synaptic paradigm which is the current understanding for learning and memory (Constantine-Paton, 1990, Malenka and Nicoll, 1999, Mori and Mishina, 1995).

NMDA receptors exist as tetramers containing two GluN1 subunits and at least one GluN2 (A-D) subunit (Lau and Zukin, 2007). The GluN2 subunits are developmentally regulated with GluN2B-containing NMDA receptors primarily expressed during development, and GluN2A-containing receptors being primarily expressed in adulthood (Hardingham and Bading, 2010). The GluN2 subunit also determines the biophysical and pharmacological properties of the receptor with GluN2A subunits having a lower affinity for glutamate, faster channel kinetics and greater open channel probability (Cull-Candy and Leszkiewicz, 2004). GluN3 (A+B) containing NMDA receptors form excitatory glycine receptors which are impermeable to Ca^{2+} and unresponsive to glutamate however their physiological role has yet to be determined. As NMDA receptors are permeable to Ca^{2+} there is a need for tight regulation of expression of the receptor as too much activation can lead to excitotoxicity (where pro-death pathways are activated due to a large influx of

Ca^{2+}), or too little stimulation can lead to signalling deficits and hyposignalling (For detailed review see Lau and Zukin (Lau and Zukin, 2007)).

1.2.2 NMDA receptor trafficking to the cell surface

1.2.2.1 ER Retention

Like many proteins, the GluN1 subunit of the NMDA receptor contains ER retention signals which act to either modulate the export of misfolded (or otherwise imperfect) NMDA receptor subunits, or to ensure GluN1 is not exported until it has assembled with the GluN2 subunit. The NMDA receptor is wholly formed within the ER thus preventing export of monomers or incompletely assembled receptor complexes. ER retention of the GluN1 subunit is dependent on alternative splicing in the C-terminus (Standley et al., 2000, Scott et al., 2001, Xia et al., 2001). The major splice variant GluN1-1 is retained within the ER because of an RRR and KKK retention motif in its C1 cassette. GluN1-2,3, are not retained because they lack this cassette. GluN1-4 is not retained because it contains a C2' cassette which contains a PDZ binding domain which has been shown to initiate premature exit from the ER through the regulation of the RRR motif (Petralia et al., 2009). Mutation of the either the KKK or RRR site to AAA results in ER retention of the GluN1 subunit and surface expression of individual GluN1 subunits is only achieved when both sites are mutated to AAA (Horak and Wenthold, 2009).

The GluN2B subunit contains only one retention motif, HLFY, which immediately follows TM4 and is required for the export of receptors from the ER (Hawkins et al., 2004). Subunits which have the HLFY motif mutated to AAAA can still form receptors with GluN1, but are retained within the ER (Hawkins et al., 2004). This indicates that forward trafficking of the receptor only occurs when the HLFY motif is accessible i.e. unmasked and if masked then there is ER retention of the NMDA receptor. Thus the motif functions as an indicator that the assembly process is complete, unlike the KKK and RRR motifs in the GluN1 subunit which are masked when correctly formed receptors are assembled. The HLFY motif is absent from the

GluN1 subunit but is present in GluN2A and GluN2B and has the sequence HLVY in GluN2C and GluN2D subunits (Hawkins et al., 2004).

Other factors can also affect the release of the GluN1 subunits from the ER. PKC phosphorylation of a serine at S896, which is next to the RXR ER retention, was reported to override the ER retention motif and elicits robust surface expression of GluN1 (Scott et al., 2001, Scott et al., 2003). This also may involve phosphorylation of an adjacent PKA site (S897) or the co-ordinated action of both PKA and PKC (Scott et al., 2001, Scott et al., 2003). These studies found; first, brain macrosomes are largely dually phosphorylated at S896 and S897 (by PKC and PKA respectively), whereas in synaptosomes the subunits are largely de-phosphorylated; Second, S896, S897 and dual SS896,897 phosphorylation can occur in early secretory compartments suggesting that PKC and PKA can phosphorylate GluN1 in the ER and /or Golgi; Thirdly, phosphomimic mutants of S897 are sufficient to override ER retention, while phosphomimic mutants of S896 sites are not. However a later study by Horak and Wenthold disputed these findings as they found mutation of the phosphosites did not result in surface expression of the GluN1 subunit (Horak and Wenthold, 2009). This could have been due to Scott et al using tac-GluN1 C-terminal tails and Horak and Wenthold using full length receptor subunits.

1.2.2.2 Trafficking to the cell surface

The mechanism of transport of the NMDA receptor from the ER to the surface membrane is still largely unknown. It is generally believed that, once released from the ER, NMDA receptors are transported to the surface of the cell where they are either inserted directly into the plasma membrane or maintained within an intracellular pool. Holding receptors within this pool allows for, upon activation of surface receptors, their rapid insertion into the plasma membrane (Perez-Otano and Ehlers, 2005).

After receptor formation, the sub-cellular localisation of the GluN2B subunit is regulated by its protein-protein interactions in the C-terminal tail. Via its PDZ domain, GluN2B binds to the adaptor proteins AP1, AP3 and AP4. These adaptor

protein facilitate cargo transport by both clathrin dependent and clathrin independent mechanisms indicating that the NMDA receptor can be trafficked from the trans-golgi network by both clathrin and non-clathrin mechanisms (Sans et al., 2000). GluN2B has also been shown to interact, between the ER and trans-golgi network, with the synaptic membrane associated guanylate kinase (MAGUK) protein SAP102 (Muller et al., 1996, Sans et al., 2005, Lau and Zukin, 2007). SAP102 is an early resident of synapses and is prevalent in the cytoplasm, suggesting that it may be involved in trafficking of membrane cargo. mPins, a ubiquitously expressed protein critical for the regulation of mitotic spindle organisation in developing cells, interacts with SAP102. Disrupting the interaction between these regulatory proteins, results in a decrease in trafficking of NMDA receptors (Sans et al., 2005). Successful interaction between mPins and SAP102 is therefore thought to be required for NMDA receptor trafficking (Sans et al., 2005).

Another protein thought to be involved in NMDA receptor trafficking is the exocyst complex component, sec8 (Prybylowski and Wenthold, 2004, Sans et al., 2003, Groc et al., 2006). Sec8 facilitates vesicle fusion to the plasma membrane although the exact mechanism of how this occurs is unknown. inhibition of the ER, Golgi and trans-golgi networks resulted in the mistargeting of Sec8 indicating Sec8 is associated with these organelles (Hsu et al., 1999, Yeaman et al., 2001, Vega and Hsu, 2001, Shin et al., 2000). Sec8 exerts its function by binding directly to the PDZ domain of SAP102 forming a complex containing the NMDA receptor (Sans et al., 2003). Use of a dominant negative form of Sec8 that lacks a PDZ binding domain has been shown to block the interaction of Sec8 with SAP102 thus preventing delivery of the NMDA receptors to the cell surface (Sans et al., 2003). Therefore NMDA receptors require both for surface expression; however GluN2B subunits lacking the PDZ binding domain have been shown to still be delivered to the cell surface and therefore can be inserted into the plasma membrane via a mechanism independent of sec8 and MAGUK (a class of scaffold proteins present in the PSD which control a number of signalling pathways), thus there is clearly further complexity waiting to be uncovered (Sans et al., 2003, Chung et al., 2004, Prybylowski et al., 2005).

Trafficking of the complex containing; GluN1, GluN2B, SAP102, Sec8 and mPins is likely to be via kinesin motors due to GluN2B being able to bind to KIF17 accessory proteins mLin 2, mLin7 and mLin10 (Guillaud et al., 2003, Setou et al., 2000, Wang et al., 2010a). Knockdown of KIF17 impairs expression and synaptic localisation of GluN2B, which is followed by an increase in GluN2A at the synapse (Guillaud et al., 2003). Therefore the complex of SAP102, sec8 and mPins modulates the transport of the NMDA receptor bound in the membrane of the carrier vesicle or tubulovesicular organelle along the dendrite.

Taken together this indicates NMDA receptors are trafficked via clathrin or non-clathrin coated vesicles from the trans-golgi network via a complex of Sec8, SAP102 and mPins which attach to kinesin motors and traffic the complex to the surface of the cell. However this may not be the only mechanism of receptor trafficking as disruption of the PDZ domain on the GluN2B subunit did not lead to total loss of NMDA receptor surface expression. These receptors must therefore be trafficked to the surface via a different mechanism.

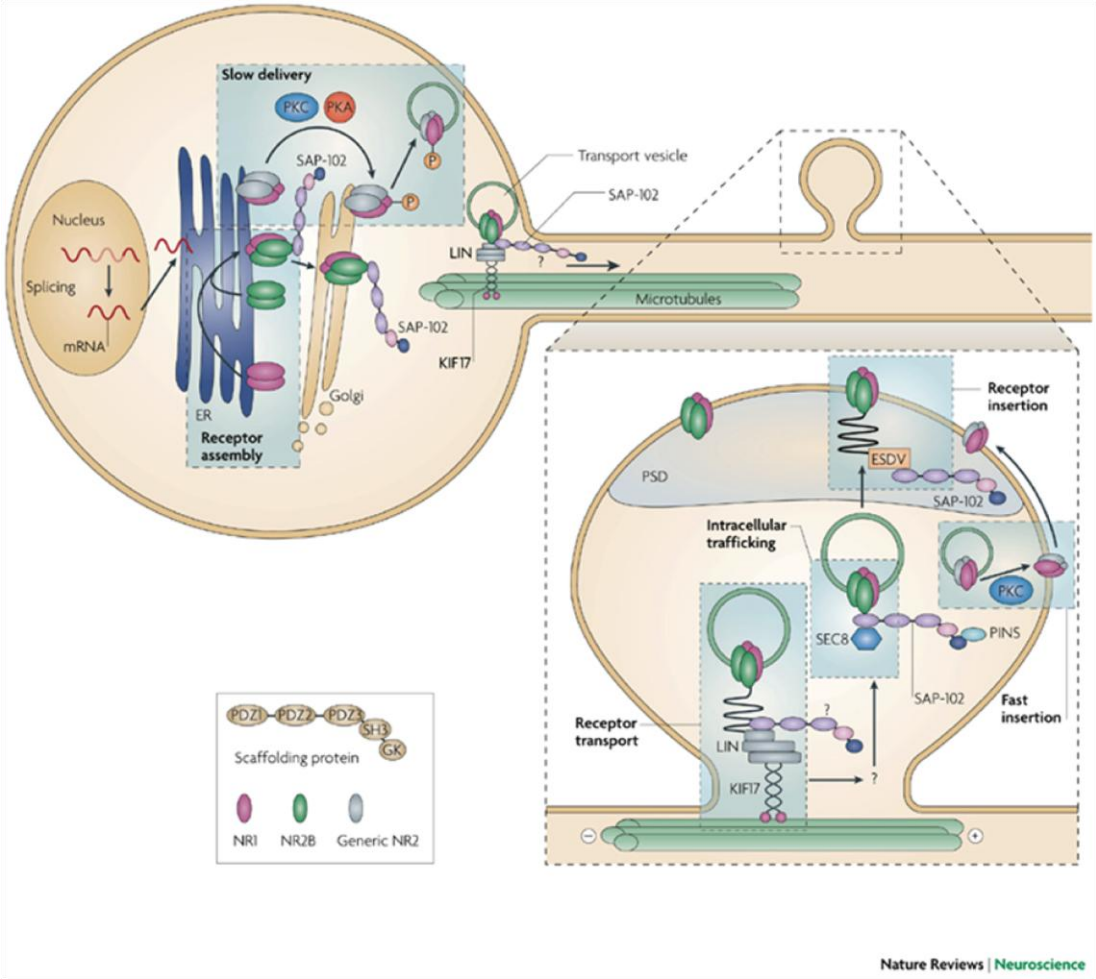

Figure 1.3 NMDA receptor trafficking from the soma and at the synapse adapted from Lau and Zukin 2007

GluN1 and GluN2 subunits are assembled into the NMDA receptor in the ER at the soma and are trafficked along microtubules via kinesin motors to a dendritic spine (top image). Receptors are inserted into the synapse via a large number of interacting proteins at either the PSD or extra synaptic sites (enlarged box).

1.2.2.3 NMDA receptor recycling and degradation

NMDA receptor recycling is a fundamental process by which neurons regulate intracellular signalling, synapse maturation and synaptic strength and involvesendocytosing receptors and either recycling them for re-insertion to the surface membrane or degrading the receptor. NMDA receptor endocytosis is a tightly regulated process and is governed by a subunit-specific set of rules. Under basal conditions NMDA receptor internalisation occurs via the clathrin-coated pit

pathway and is mediated by interactions between tyrosine-based internalisation motifs contained within the GluN2 C-terminus and the adaptor protein 2 (AP2).

GluN2A and GluN2B contain different internalisation motifs in their distal C-termini, which regulate internalisation at different rates (Lavezzari et al., 2004). GluN2B endocytosis is more rapid than that of GluN2A and is mediated by the interaction between the YEKL motif and AP2 (Roche et al., 2001), whereas GluN2A is mediated by a dileucine motif (LL) (Lavezzari et al., 2004). NMDA receptor subunits also contain proximal endocytic motifs; GluN1 subunits contain two independent signals (YKRH and VWRK); GLuN2B contains one (YWQF); and GluN2A also contains one (YWKL) all of which are necessary and sufficient to drive the internalisation of NMDA receptors (Scott et al., 2004). While the proximal and distal signals contribute in an additive manner to endocytosis they exert distinct effects on post-endocytic trafficking. Proximal C-terminal motifs in GluN2B direct receptors to recycling endosomes whereas GluN2A and GluN1 proximal signals direct internalised receptors to degradative endosomes (Scott et al., 2004). By coding for degradation or recycling, endocytic motifs provide a powerful means to control NMDA receptor synaptic abundance by co-ordinating intracellular trafficking.

Due to the nature of the NMDA receptor there is tight regulation at key steps during its surface expression in order to ensure it is expressed correctly and in the required amount. ER retention ensures misfolded and incorrectly assembled receptors do not get expressed at the surface. Internalisation motifs ensure regulation of signalling, maturation and cell death. Although there is a general understanding of the mechanism behind these processes the full mechanism has yet to be elucidated, as knockdown studies of key trafficking components of the NMDA receptor do not inhibit surface expression indicating there is another way of trafficking of NMDA receptors.

1.3 DISC1 and NMDA receptors

Studies in the Millar lab have revealed a direct interaction between the GluN1 subunit and DISC1 (S. Mackie & K. Millar, unpublished). This was originally found via

a peptide array and was later confirmed via co-immunoprecipitation. Furthermore DISC1 plays a critical role in regulating excitatory synaptic function through TNIK and kalirin (Hayashi-Takagi et al., 2010, Wang et al., 2010b) and loss of DISC1 *in vitro* leads to increased NMDA receptor current densities in cortical cultures, resulting in an increase in GluN2A containing NMDA receptors (Wang et al., 2010b). Overexpression of GluN2A in the forebrain leads to deficits in certain-forms of long term memory and long term depression, which could link DISC1 dependent increases in GluN2A to dysfunction of synaptic and cognitive processes (Cui et al., 2013).

DISC1 has also been shown to regulate glutamate release from presynaptic terminals (Maher and LoTurco, 2012). Studies showed that, when a truncated form of DISC1 is transfected into cells, it enhances the frequency of mEPSC and disrupts the synchronous nature of evoked glutamate release. Also the expression level of DISC1 in presynaptic neurons correlates with the probability of glutamate release, which is interesting as certain splice variants of DISC1 have been found to have higher expression in some patients with schizophrenia (Nakata et al., 2009), which could then lead to the misregulation of glutamate firing. Taken together there is an emerging and interesting link between DISC1 and NMDA receptors, coupled with DISC1s role in subcellular trafficking DISC1 could play an important role in NMDA function.

As DISC1 is known to interact with many trafficking molecules and has been implicated in trafficking via dynein and kinesin, while NMDA receptors are known to be trafficked from the Golgi to the surface via kinesin, I hypothesised that DISC1 may be involved in NMDA receptor trafficking. This thesis describes my investigation of this hypothesis.

1.4 NDE1 function and in mental illness

1.4.1 NDE1 in mental illness

Nuclear distribution element 1 (NDE1) and its orthologue NDE-like1 (NDEL1) are a pair of highly similar coiled-coil proteins which have been shown to play significant roles in a variety of vital cellular functions including mitosis, neuronal migration as well as microtubule organisation during brain development (Toyo-Oka et al., 2005, Hirohashi et al., 2006b, Shu et al., 2004, Hirohashi et al., 2006a). Both NDE1 and NDEL1 were identified in mammals through their interaction with lissencephaly 1 (LIS1), a gene which, when mutated, results in human lissencephaly (Feng et al., 2000, Feng and Walsh, 2004, Niethammer et al., 2000, Feng and Walsh, 2001). Lissencephaly is a rare but severe brain malformation which results in the appearance of a smooth surface of the brain rather than the characteristic "folds". Mutations of the LIS1 gene cause a disruption in binding between LIS1 and NDEL1 which results in an abnormal pattern of cortical development and the subsequent appearance of a 'smooth brain' on MRI or post-mortem (Feng and Walsh, 2001).

NDE1 and NDEL1 are thought to have evolved from a common ancestral gene and are homologous proteins as ~60% of the amino acid sequence identity of these protein are the same. Due to these similarities it has previously been presumed that they both undertake identical functions. Whilst this can be the case in some instances e.g. maintaining the correct position of the Golgi, for other functions their parity has often been presumed without full investigation of the role of the other protein. (Efimov and Morris, 2000, Feng et al., 2000). Although the two proteins have overlapping functions and can to some extent compensate for the loss of one another, there is evidence suggesting that they also have different functions within the cell. In particular, they undertake distinct roles in the dynein-related regulation of mitotic checkpoints (Vergnolle and Taylor, 2007), and also have different binding sites on the dynein protein complex, although these do overlap. Furthermore, NDEL1 knockout mice are embryonically lethal whereas NDE1 knockout mice maintain viability to birth (Sasaki et al., 2005).

NDE1 and NDEL1 have not been highlighted in genome-wide association studies using samples from psychiatric patients, thus there are no common ancient causal variants in these genes. However both NDE1 and NDEL1 have been implicated in a number of mental health disorders (such as schizophrenia, bipolar affective disorder, ADHD) by genetic association (Hennah et al., 2007, Tomppo et al., 2009). There was initially little evidence linking disruptions in NDE1 with schizophrenia. However, subsequently NDE1 was implicated in the development of schizophrenia in a study of the Finnish population (Hennah et al., 2007). To begin with the authors didn't find any direct link between NDE1 and schizophrenia, but once the dataset was conditioned on the presence of a previously identified risk haplotype in DISC1, a significant link between NDE1 and the development of schizophrenia was detected (Hennah et al., 2007).

In a separate study, a Caucasian-American schizophrenia cohort found no direct association of NDE1 with schizophrenia but did find evidence for an interaction between NDE1 and DISC1 on schizophrenia susceptibility, which, interestingly depended on the status of a common variant in DISC1 (C704) (Kamiya et al., 2006). These studies further strengthen the link between mutations within NDE1 and mental illness.

Tentative associations between NDEL1 and schizophrenia have been proposed via an interaction between an independently significant mutation to the CIT gene, which encodes a protein kinase involved in cell division. However an association between NDEL1 and CIT have not been widely replicated and therefore this evidence should be interpreted with caution. (Nicodemus et al., 2010, Ikeda et al., 2008, Kahler et al., 2008, Numata et al., 2008, Rastogi et al., 2009).

A number of mental illnesses have been associated with 'relatively frequent' copy number variations (CNVs) (both duplication and deletions) at the locus of the NDE1 gene (16p13.11) including autism (duplication) (Ullmann et al., 2007), ADHD (duplication) (Williams et al., 2010) and schizophrenia (deletion and duplication) which had a penetrance of 2 and 7.4 (Need et al., 2009, Sahoo et al., 2011). There

are a number of other genes located at the locus 16p13.11 but NDE1 is the most likely candidate for disruption of normal function causing the aforementioned mental illnesses due to the role of NDE1 within the cell and due to its interaction with DISC1 (Bradshaw et al., 2013). Taken together these observations highlight NDE1 as a possible risk factor for mental illness. This, coupled with its interaction with DISC1, indicates the importance of studying the potential role of NDE1 in influencing disease risk. There is less evidence for a link between NDEL1 and mental illness, therefore this introduction will focus mainly on NDE1 function and its link to DISC1.

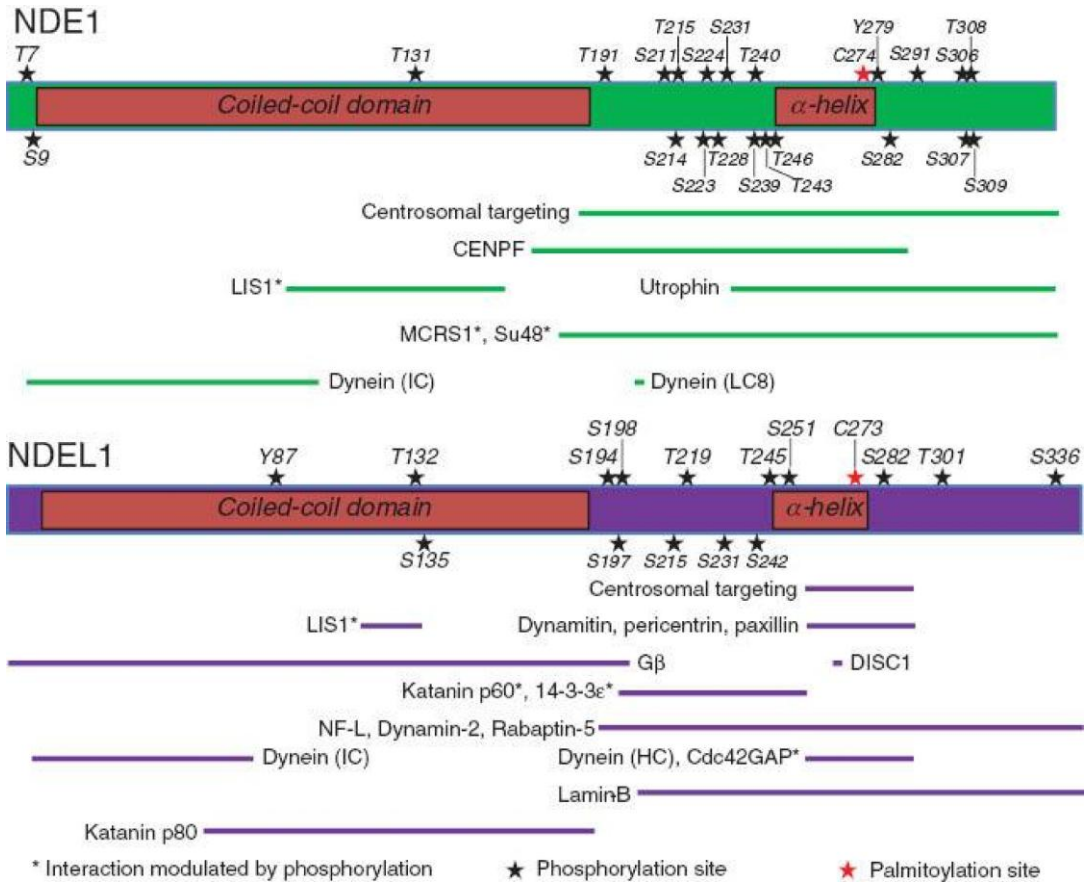

Figure 1.4 Structure, Binding, phosphorylation and palmitoylation sites of NDE1 and NDEL1 adapted from Bradshaw et al 2013

The structure of NDE1 and NDEL1 is similar as both contain a coiled-coil domain and an α-helix. They have largely different phosphorylation sites (locations shown as stars) and different binding/interaction sites with different proteins (represented by solid lines)

1.4.2 NDE1 function

Via its interaction with cytoplasmic dynein, NDE1 is known to play a role in a wide variety of cellular processes. Along with its binding partners LIS1 and NDEL1, NDE1 regulates cytoskeletal organisation, intracellular transport, membrane trafficking, and cell signalling. As described in 1.1.4.2 cytoplasmic dynein is a multisubunit complex that functions as a minus end-directed microtubule motor and plays critical roles in a variety of eukaryotic cellular functions, including retrograde axonal transport (Stehman et al., 2007, Paschal and Vallee, 1987), directed cell migration (Dujardin et al., 2003) and numerous aspects of mitosis. Dynein function is regulated by several protein complexes e.g. LIS1/NDE1/NDEL1, dynactin (Lam et al., 2009, Allan, 2011). NDE1 binds directly to the intermediate chain of dynein (Stehman et al., 2007, Lam et al., 2009) where it competes with dynactin for binding to dynein. Once bound NDE1 recruits LIS1 which results in increased transport along microtubules (McKenney et al., 2010). The NDE1/LIS1/Dynein complex is essential for the correct organisation and function of the microtubule network during neurite outgrowth and neuronal migration (Wynshaw-Boris and Gambello, 2001, Wynshaw-Boris, 2007) showing NDE1 plays a critical role in cellular functions via regulation of dynein function.

The intracellular positioning of organelles is dependent on correct dynein function. Using siRNA to reduce the levels of NDE1, Lam et al showed that the Golgi complex became fragmented and mispositioned (Lam et al., 2009). This NDE1 depletion also had an effect on the positioning of lysosomes and early endosomes, as LAMP1 (a marker for lysosomes) and EEA1 (early endosome marker) distribution was also altered (Guo et al., 2006, Lam et al., 2009). The effect of NDE1 knockdown on Golgi scattering and on the positioning of the lysosomes was reversed when NDE1 was reintroduced into the cell (Lam et al., 2009), thus indicating that NDE1 plays an important role in dynein-dependent positioning of cellular components.

Studies into NDE1 using knockout mice uncovered several roles for NDE1 within neurons. NDE1 knockout mice displayed large reductions in several regions of the brain (neocortex, cerebellum, thalamus) indicating NDE1 is essential for the correct

development of the brain (Feng and Walsh, 2004). The knockout mice also display a thinning of cortical neurons in the superficial layers but no change in the deeper cortical layers. BrdU birthdating was used to determine if the observed cortical layering abnormalities were due to impaired neurogenesis, rather than abnormal neuronal migration. The authors found no changes in BrdU staining of the deeper layers of the cortex at E13, however BrdU-positive neurons stained at E15 or E17 show a reduced distance of migration and a more scattered distribution within the cortical layers in NDE1 knockout mice. Furthermore there was a decrease in the overall number of neurons, indicating a reduced neuronal proliferation rate in cortical progenitor cells (Feng and Walsh, 2004). The authors also found NDE1 to play a role in mitosis after observing an increase number of cortical progenitors in metaphase or anaphase in NDE1 knockout mice (Feng and Walsh, 2004). This was replicated by another group who observed mistargeted spindles after antibody disruption of NDE1. Additionally the authors observed the cells did not progress from metaphase, due a lack of dynein function as this relies on correct NDE1 function (Stehman et al., 2007). Furthermore NDE1 knockdown by siRNA in HeLa cells causes an increase in duration of mitosis of 197% indicating a role for NDE1 in mitotic progression in HeLa cells (Lam et al., 2009).

Mutations within NDE1 have been shown to cause (co-segregate) with microlissencephely. Alkuraya et al showed that two mutations within NDE1 caused a frameshift mutation resulting in a truncated protein lacking the dynein interaction site (Alkuraya et al., 2011). The authors found a lack of expressed NDE1 in patient lymphoblastoid cells which indicated the proteins are degraded and therefore there is a large reduction in mitotic spindle organisation, interkinetic nuclear migration and neuronal migration resulting in the aforementioned microlissencephaly (Alkuraya et al., 2011).

1.4.3 Posttranslational modifications

Posttranslational modifications are essential modifications which regulate the activity of proteins thereby controlling their function. Phosphorylation is a well studied form of posttranslational modification where a phosphate group is added to

a protein which “activates” or “deactivates” the protein. NDE1 has a number of experimentally determined phosphorylation sites which modulate its subcellular localisation and protein interactions (Yan et al., 2003, Hirohashi et al., 2006b, Hirohashi et al., 2006a, Bradshaw et al., 2011). Hirohashi et al identified several phosphosites which were found to modulate the interaction of the centrosomal protein Su48 and NDE1 (Hirohashi et al., 2006b). Using bioinformatics the group identified 6 potential phosphosites (T191, T215, T228, T243, T246 and S282), all within the C terminal flexible linker region, and created phosphomimic and phosphomutant versions of these sites to test whether the interaction between of NDE1 and Su48 is altered. The phosphomimic NDE1 had significantly reduced interaction with Su48 (a centrosomal protein essential for cell division (Wang et al., 2006) and the phosphomimic version of NDE1 had altered subcellular localisation when analysed by florescence microscopy. The T246 site was later shown to be phosphorylated at G2/M phase in cell division by Cdk1 and, using T246A mutant NDE1 cells, arrest at the G2 phase suggested phosphorylation of T246 is essential for G2/M transition, thus providing an explanation for how knockdown of NDE1 results in neuronal proliferation deficits (Hirohashi et al., 2006b)(Hirohashi et al., 2006a)(Alkuraya et al., 2011) NDE1 is also a known target of the cAMP-dependent kinase PKA (Bradshaw et al., 2008, Bradshaw et al., 2011) as will be discussed in the next section.

Taken together the literature shows that NDE1, through its interaction with dynein plays a vital role in mitosis and cell division, correctly positions organelles, regulates intracellular trafficking, neuronal migration and neurogenesis. Post-translational modifications have been shown to provide a way in which NDE1 function can be regulated. However the full function of this protein has yet to be determined.

1.4.4 Interaction with DISC1

NDE1 is a known DISC1 interactor (Millar et al., 2003, Brandon et al., 2004, Burdick et al., 2008, Bradshaw et al., 2008, Bradshaw et al., 2009). Together they regulate neurite outgrowth and may, as NDE1 and DISC1 are both known trafficking molecules, interact to regulate aspects of intracellular trafficking. NDE1 and DISC1

were first identified as potential interacting partners through yeast 2 hybrid screens of DISC1 (Millar et al., 2003, Brandon et al., 2004). Direct interaction between these proteins was shown by Burdick et al where the authors co-immunoprecipitated both exogenous NDE1 and DISC1, and endogenous NDE1 and DISC1 from SH-SY5Y (Burdick et al., 2008). This interaction was later confirmed as a direct interaction by co-immunoprecipitation of in vitro transcribed and translated DISC1 and NDE1 (Bradshaw et al., 2009). Together these data show that there is a robust interaction between DISC1 and NDE1.

DISC1 also interacts with the cAMP phospodiesterase PDE4, which complexes with DISC1, NDE1, LIS1 and NDEL1 at the centrosome, suggesting a potential role for cAMP dependent regulation of NDE1/LIS1/NDEL1 by DISC1/PDE4. In addition PDE4 co-precipitates with dynein intermediate chains (Bradshaw et al., 2008) which indicates a potential involvement in regulating aspects of intracellular trafficking. Indeed, bioinformatic analysis of NDE1 identified two potential phosphosites, at positions T131 and S306, which may be regulated by PKA. Bradshaw et al used an antibody raised against phosphorylated PKA motifs in conjunction with IBMX and forskolin, to increase intracellular cAMP levels and stimulate PKA activity. This analysis determined that NDE1 can be phosphorylated by PKA in a DISC1/PDE4-dependent manner (Bradshaw et al., 2008). Phosphorylation of T131 modulates NDE1 binding to LIS1, as there is a significant reduction in NDE1/LIS1 binding when a phosphomimic form of NDE1, T131E-NDE1, is used for co-immunoprecipitation experiments. Consistent with this, after IBMX/forskolin treatment there is a significantly reduced binding between endogenous NDE1 and LIS1. However, under these conditions there is increased NDE1/NDEL1 binding, indicating that phosphorylation/dephosphorylation at T131 may act as a switch to determine whether NDE1 binds to LIS1 or NDEL1 and DISC1/PDE4 may regulate this process via local cAMP gradients (Bradshaw et al., 2011). It could be speculated that this mechanism also regulates dynein activity: As described earlier (1.4.2) NDE1/LIS1 interaction activates dynein and this interaction is needed for several key

intracellular processes. The modulation of NDE1/LIS1 binding by cAMP could therefore regulate dynein activity.

Finally NDE1 has been shown to be present at the synapse where it co-localises with PDE4B. Mouse hippocampal neurons were transfected with NDE1 and PDE4B and NDE1 was found to accumulate in proximal axons suggesting a potential role in determining axon polarity. Further to this, co-localisation between NDE1 and PBE4B was observed in dendritic spines. Coupled with evidence of DISC1 co-localisation with PSD95 (Bradshaw et al., 2011) and studies showing DISC1 localisation in the PSD via electron microscopy (Kirkpatrick et al., 2006) DISC1/NDE1/PDE4 may function together at the post-synapse.

Altogether then, NDE1 is critical for a number of intracellular processes and via its interaction with LIS1 and Dynein can regulate many trafficking processes. Further to this NDE1 has been implicated as a risk factor for psychiatric illness and binds directly to the risk factor DISC1.

1.5 TRAK1 function and interaction with DISC1

1.5.1 TRAK1 Discovery and function

Trafficking-protein-Kinesin-binding-1 (TRAK1) in a trafficking protein involved in the transport of a range of different cargoes such as mitochondria, early endosomes and $GABA_A$ receptors (Gilbert et al., 2006, Webber et al., 2008, Brickley et al., 2005, Brickley and Stephenson, 2011). Genetic screens in *Drosophilla* identified two proteins critical for mitochondrial transport in neurons, named Milton and Miro (Mitochondrial Rho GTPase)(Stowers et al., 2002, Guo et al., 2005). In mammals there are two orthologes of Milton named TRAK1 and TRAK2 (Brickley et al., 2005, Smith et al., 2006). The TRAKs act as motor adaptor proteins which connect kinesin motors to the mitochondria anchored protein Miro, thereby linking motors to mitochondria for movement within the cell (MacAskill et al., 2009, Wang and Schwarz, 2009). The TRAKs interact with miro via their C-terminal domain, with the N-terminal forming a coiled-coil region which is also found in several kinesin and dynein interacting proteins (Glater et al., 2006, Stowers et al., 2002).

TRAK1 and TRAK2 associate with kinesin both *in vitro* and *in vivo*. This indicates a strong association between these two proteins and kinesin, and provides evidence that TRAK1 and TRAK2 are species orthologues of Milton (Brickley et al., 2005). The use of a dominant negative TRAK2 (DN-TRAK2) species resulted in the inhibition of both TRAK1 and TRAK2. Brickley and Stephenson used this to inhibit both TRAK1 and TRAK2 association with the mitochondrial motor KIF5C, which resulted in a significant decrease in mobile axonal mitochondria in hippocampal neurons (Brickley and Stephenson, 2011). To determine any differences in TRAK1 and TRAK2 function the authors also used shRNAi to individually knock down TRAK1 or TRAK2 protein expression. TRAK1 knockdown resulted in a significant decrease in the number of mobile mitochondria in both the anterograde and retrograde direction, similar to what was observed in the DN-TRAK2 study, which was rescued by exogenous expression of either TRAK1 or TRAK2. Interestingly TRAK2 knockdown by shRNAi caused no significant effects on axonal mitochondrial trafficking, indicating that TRAK2 might be predominantly localised in a different neuronal location e.g.

soma or dendrites, or that the two TRAK proteins may have different functions within the cell (Brickley and Stephenson, 2011). The difference in effect of mitochondria mobility after knockdown was subsequently shown to be due to occupation of different in cellular locations. Van Spronsen et al showed that TRAK1 is predominantly localised to the axon whereas TRAK2 is predominantly localised to the dendrites, explaining the differences in KD between TRAK1 and TRAK2 (van Spronsen et al., 2013).

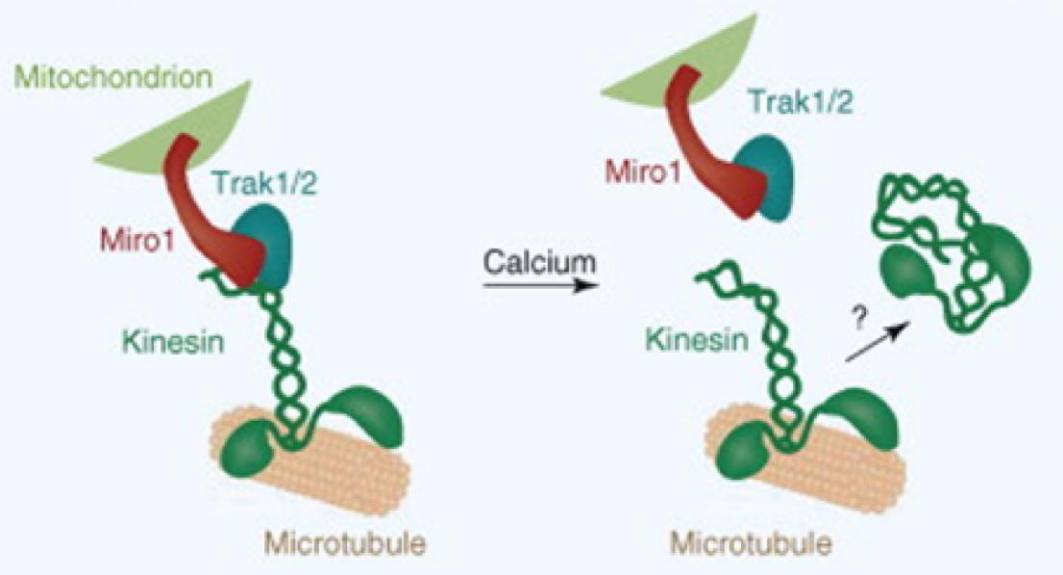

Figure 1.5 TRAK1 and TRAK2 interaction with kinesin for cargo transport, adapted from MacKaskill and Kittler 200

In mitochondrial trafficking TRAK1 binds to Miro1 which is bound to the mitochondria. TRAK1 links this with kinesin to move the mitochondria through the cell via microtubules, calcium is used to inhibit the movement possibly by altering the kinesin structure leading to the de-coupling from TRAK1. TRAK1 can also bind other proteins and transport the cargo to different locations within the cell via kinesin motors.

TRAK1 and TRAK2 have also been shown to bind to the dynein motor complex. Mass spectrometry and subsequent western blotting showed an interaction between the TRAK proteins and a number of components of the dynein complex (van Spronsen et al., 2013). Co-IP experiments determined binding between the p150Glued domain of dynactin and both TRAK1 and TRAK2, providing further evidence for the interaction between these proteins (van Spronsen et al., 2013).

Not only can TRAK1 and TRAK2 bind to the dynein complex, van Spronsen et al showed, using an inducible cargo-trafficking assay which labels static cargoes which can then be linked to trafficking molecules via administration of rapalog (a small molecule used to trigger binding of motor proteins of interest to mitochondria), that TRAKs mediate dynein-dependent motility. When KIF5 is overexpressed there is an accumulation of cargoes at the cell periphery and if dynein is overexpressed there is an accumulation of cargoes in more central regions. TRAK1 or TRAK2 overexpression induced a significant redistribution of static cargoes to both the cell periphery and to central regions, indicating TRAK proteins mediate both dynein and kinesin motor activity. This was further confirmed when knockdown of KIF5B resulted in a large accumulation of cargoes in central regions, similar to that observed when dynein is overexpressed. This indicates that, in the absence of kinesin, dynein actively transports TRAKs. Similarly, blocking dynein binding to TRAK1 or TRAK2 using HA-p50 resulted in a random distribution of cargoes, and co-expression with KIF5B re-distributed the cargoes to the cell periphery (van Spronsen et al., 2013). This demonstrates that TRAK1 and TRAK2 not only bind to dynein but they are also able to modulate its motor activity.

1.5.2 TRAK1 in receptor trafficking

TRAK1 was recently identified as the mutated gene in *hyrt* mice, an animal model of hypertonia (Gilbert et al., 2006). The authors showed that the hypertonia observed in the *hyrt* mice was alleviated by both the administration of $GABA_A$ or $GABA_B$ agonists indicating that a dysfunction of GABA receptors is responsible for the hypertonia. TRAK1 immunoprecipitated with the α1 subunit of the $GABA_A$ receptor indicating an association of these proteins. The mutation in *TRAK1* results in a frameshift in the C-terminus, leading to the formation of a truncated protein, TRAK1 1-824 which immunoprecipitated with the α1 subunit of the $GABA_A$ receptor in similar amounts, indicating that mutant TRAK1 has no apparent affect on the association of $GABA_A$ and TRAK1 (Gilbert et al., 2006). Based on the administration of GABA agonists reliving the observed hypertonia in the mouse model the authors hypothesised that TRAK1 plays an essential role in the trafficking of GABA receptors.

TRAK1 interaction with the $GABA_A$ receptor was further supported by yeast-2-hybrid interaction assays (Beck et al., 2002, Stephenson, 2013). The location of the interaction between TRAK1 and the GABAa receptor has yet to be determined. However Anne Stephenson showed TRAK1 to interact with the β3 subunit of $GABA_A$ (Stephenson, 2013) receptors whereas Gilbert et al (Gilbert et al., 2006) observed an interaction between the α1 subunit and TRAK1. However the disparity between these results could be explained by the antibodies used in the experiments. Gilbert et al used an anti-α1 subunit antibody, which given that the $GABA_A$ receptor contains both α1 and β3 subunits does not necessarily mean that TRAK1 associated with the α1 subunit. In any case, these studies provide evidence for additional functions of TRAK1 other than mitochondrial trafficking via kinesin and dynein.

1.5.3 TRAK1 in endocytosis

TRAK1 however has been shown to regulate the transport of other cargoes including early endosomes (Kirk et al., 2006, Webber et al., 2008). Although originally thought to predominantly traffic and localise to mitochondria Webber et al showed endogenous TRAK1 was not primarily localised to the mitochondria as the group found only a 39.4% overlap with the inner mitochondrial membrane marker, TIM23 in HeLa cells (Webber et al., 2008). They went on to show a 64.3% overlap with the early endosome marker EEA1, a 21.8% overlap with the late endosome marker LAMP2, and a 21.2% overlap with the ER marker KEDL (Webber et al., 2008). Indicating that TRAK1 is predominantly localised to early endosomes and therefore may have additional roles within the cell other than linking mitochondria to kinesin motors.

This indicates that TRAK1 may play a role in endosome trafficking. Using epidermal growth factor-induced (EGF) degradation of the EGF receptor (EGFR) (a widely used model for studying endocytic trafficking) Webber et al (Webber et al., 2008) showed overexpression or KD of TRAK1 inhibits EGF induced EGFR degradation but not EGFR endocytosis. Using [I^{125}]EGF they showed that cells expressing TRAK1 had similar amounts of internalised [I^{125}]EGF compared to control cells, indicating that TRAK1 has no significant effect on endocytosis. Using a degradation assay, the cells were

incubated with [I^{125}]EGF for 10 minutes and chased for 1,2, or 3 hours to allow for degradation. It was shown that 69.7% of internalised EGF was degraded in control, and 39.9% of internalised EGF was degraded in TRAK1 expressing cells. Similarly TRAK1 KD resulted in no change in EGF internalisation but there was a statistically significant decrease in degradation. Using a "pulse-chase" trafficking assay they went on to show that TRAK1 impedes endosome to lysosome trafficking, so taken together this supports a functional role for TRAK1 in regulating the trafficking of internalised EGF-EGFR complexes to the lysosome (Webber et al., 2008).

1.5.4 TRAK1 mental illness and DISC1 interaction

There is little evidence linking disruptions in TRAK1 to mental illness . One study found a non-synonomys mutation of TRKA1 (via poly-phen2 analysis) in a patient with schizophrenia TRAK1 H678R. Analysis of the mutated region predicted that the observed mutation could cause a damaging alteration of protein function. Although the authors did not specifically discuss the mutation in TRAK1 and how it may cause schizophrenia they did draw attention to TRAK1 mutant mice and $GABA_A$ dysfunction. Additionally the authors found a correlation between *de novo* mutations and disruptive amino acid changes therefore there is a high likelihood for pathogeneity (Xu et al., 2011). As TRAK1 plays a critical role in mitochondrial trafficking any disruption to its function could cause interference to mitochondrial movement and therefore upset the homeostasis within cells. This could in turn lead to altered synaptic transmission as energy demands at the synapse are great (Harris et al., 2012). However although there is only tentative evidence likening TRAK1 to mental illness as TRAK1 has only relatively recently been found to interact with DISC1 and its function is still being analysed more evidence is needed before any firm conclusions can be made about its role in mental illness

Recently an association between TRAK1 and DISC1 has been identified. Co-IP of both exogenous and endogenous DISC1 and TRAK1 in mammalian cell lines indicates a robust association, but not necessarily a direct interaction, between

these two proteins, however the association between these two proteins was not confirmed in brain at that time due to lack of a suitable antibody for TRAK1 (Ogawa et al., 2013). TRAK1/DISC1 association has since been confirmed in mouse brain (F. Ogawa & K. Millar, unpublished). Overexpression of TRAK1 causes mitochondrial clustering in the majority of cells (Brickley et al., 2005). When DISC1 is co-expressed with TRAK1, DISC1 expression at the mitochondria becomes more pronounced and isolation of mitochondria from transfected COS7 cells showed that TRAK1 recruits DISC1 to the mitochondria (Ogawa et al., 2013). Therefore DISC1 and TRAK1 clearly associate, with the function of this association, and consequences of disrupting this function still to be fully investigated.

Sequence variants in DISC1 have been shown to alter its protein-protein interactions and its function. DISC1-37W overexpression results in mitochondrial clustering in approximately 50% of transfected cells in a pattern very similar to that induced by TRAK1 overexpression (Ogawa et al., 2013). The location of 37W lies within the TRAK1 association site on DISC1 and co-immunoprecipitation studies revealed a 50% increase in TRAK1 association with DISC1-37W when compared to WT-DISC1 (Ogawa et al., 2013). This indicates that mutations within DISC1 may alter its interaction with TRAK1 and could conceivably alter overall function of the DISC1/TRAK1 complex. Furthermore DISC1 has been shown to increase anterograde mitochondrial movement when overexpressed in mouse hippocampal neurons. However, DISC1-37W did not promote anterograde movement, nor did it increase total mitochondrial density (Ogawa et al., 2013). Thus the 37W variant impairs the ability of DISC1 to stimulate anterograde mitochondrial transport. This may be due to the effect of DISC1-37W at the mitochondria where it induces altered interactions within the mitochondrial transport complex (Ogawa et al., 2013).

2 Methods

2.1 Bioinformatics

2.1.1 DNA sequence analysis

The sequences for all the expression plasmids used in this study were validated by direct sequencing. Plasmid DNA sequences were obtained using the method described in 2.6.2 and aligned to the reference sequence using standard nucleotide BLAST algorithm:

http://blast.st-va.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome

2.2 Cell Culture

2.2.1 Maintenance of cell lines

All cell culture work was carried out in an Envair Bio2+ class II safety cabinet, under contamination level 1.

Cell lines were grown in T75 or T175 Cell Star (Grenier Bio-one) flasks and maintained in an incubator at 37 °C and 5 % CO_2. All the media and buffers, unless otherwise stated, were obtained from Gibco, Invitrogen.

Cells were grown in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% Foetal Bovine Serum (FBS) and split when they reached 90-95% confluency. To split the cells, old media was aspirated and the cells were washed twice with DPBS. Tryple-express was diluted in a 1:1 ratio with Versene and 15 mL was added to the cells. After incubation for 2-5 minutes the cells were aided in detachment by gently tapping the flask and 3 volumes of DMEM/FBS were added, then the cells were transferred to a falcon tube and spun at 1000rpm for 5minutes. The cells were resuspended in 10 mL of fresh DMEM/FBS, if necessary the cell concentration was determined as described in 2.2.2. If the cells were needed for future use they were passaged by dilution of the cell suspension in a 1:25 ratio in a new flask containing fresh DMEM/FBS.

2.2.2 Cell Counting

To determine the cell concentration of cell suspensions the cells were harvested as described in 2.2.1 and counted using an Improved Neubauer Heamocytometer. First the haemocytometer and coverslip were washed in 70 % ethanol and dried. The coverslip was then humidified and applied to the haemocytometer. The cell suspension was gently mixed and a small volume was applied to the two chambers of the haemocytometer. The cells were counted in a $0.1mm^3$ area of each chamber, and then an average cell count was calculated and multiplied by 10^4 to obtain the cell concentration expressed in cells/mL.

2.2.3 Cell Plating

For cells to be transfected for immunocytochemistry they were seeded on dry heat sterilized glass cover slips (VWR) in sterile 12-well plates (Corning). The cells were first harvested and counted as described in 2.2.1 and 2.2.2, then diluted to the final concentration of $3x10^5$ cells/mL before equal volumes of cell suspension were added to each well. Cells were placed in an incubator and transfected 16-18 hours after plating.

2.2.4 Transfection

Transfection of COS-7 cells was carried out using the liposome-based reagent Lipofectamine-2000 (Invitrogen). For transfection of COS-7 cells; on the afternoon of the day before, cells were plated in multi-well plates as described in 2.2.3. Cells were transfected as according to the manufacturer's protocol, although the DNA concentration was adjusted, to 2.5 µg maximum, as the protocol suggests.

2.3 Primary Culture

2.3.1 Dissection and preparation of neurons

All buffers, media and supplements used for the preparation and maintenance of the primary cultures were obtained from Gibco, Invitrogen. Two days before dissection glass coverslips were sterilized in 100 % ethanol overnight with end-over-end agitation at 4 °C. Then the coverslips were washed in fresh 100 % ethanol twice, dried and heat sterilized. To allow the neurons to adhere, the day before the neuron preparation the coverslips were coated in Poly-D-Lysine by applying the Poly-D-Lysine solution to individual coverslips in a 12 well plate, and left overnight at 37 °C. The coverslips were then washed twice in distilled water and left to dry before seeding neurons on them as described below.

Eighteen days post-fertilization pregnant C57 mice were sacrificed by an experienced animal facility technician then handed to me for dissection. Embryonic day 18 (E18) foetuses were extracted using sterile surgical tools from the mother's uterus and immediately placed in ice cold dissection buffer (DB – 3:2 ratio of triple express:versine to make a 0.1 % tripsin containing solution). The brains were removed from the embryos and placed in fresh DB and dissected under a Leica M26 microscope with a Fiber-Lite MI-150 high intensity illuminator. After removal of the meninges, the cortices were separated and the hippocampus was removed and stored in ice cold DB until all the brains had been dissected.

Once all of the hippocampi had been dissected they were placed in 10 mL of 0.01% trypsin (diluted with DB) for 45minutes at 37 °C and were manually inverted every 10-15minutes. After trypsinisation the trypsin solution was removed using a plastic pasteur and the neurons were re-suspended in 2 mL DMEM supplemented with 10%FBS, the volume was made up to 10 mL and the neurons were passed through a 40 µm cell strainer (BD bioscience) and spun at 1000rpm for 5 minutes. The medium was removed and the neurons were re-suspended in 10 mL of Neurobasal medium supplemented with B27 and Glutamax and counted as described in 2.2.2. The

neurons were seeded at a concentration of $3x10^6$ cell/mL in 1 mL on Poly-D-Lysine treated coverslips and maintained at 37 °C with 5% CO_2.

2.3.2 Maintenance

The neurons were fed with fresh Neurobasal medium every 7 days. As the neurons were seeded at 1 mL an additional 1 mL was added the following day. Every 7 days after that 1 mL was removed and 1 mL of fresh medium was added. Cells were transfected between days in vitro (DIV) 18-20 and stained the following day.

2.3.3 Transfection of primary cultures

Transfection of primary neurons were carried out using the liposome-based reagent Lipofectamine-2000 (Invitrogen). Primary neurons were obtained and maintained as described in 2.3.1 and 2.3.2 and transfected at DIV 18, and the DNA lipofectamine complexes were assembled in 200 µl of warm neurobasal media supplemented with glutaMAX only, 4 µl of Lipofectamine 2000 and 2-3 µg of DNA. 1 hour before the DNA lipofactamine complexes were added the media was removed from the wells and kept in a 50 mL falcon tube and replaced with fresh neurobasal media without B27 serum. The DNA lipofectamine 2000 complexes were added and left for 3-4 hours before removing and replacing with the old pre-conditioned medium.

2.4 Drug and cell treatments

2.4.1 Mitotracker RED

MitotrackerRED is a cell permeant red fluorescent protein which diffuses across the plasma membrane and accumulates in active mitochondria, where it is retained after fixation and permeabilisation, allowing the visualisation of mitochondria. MitotrackerRED was added to the cells approximately 24 hours after transfection where the MitotrackerRED was diluted to 50 mM in DMSO and added to the media for 20-30 minutes. Cells were washed twice in warm PBS before being processed as described in 2.5.

2.4.2 NMDA antagonists

DL-AP5 is a selective NMDA receptor antagonist. It acts as a competitive inhibitor of the glutamate binding site of the NMDA receptor and therefore inhibits receptor opening.

MK-801 is a collective non-competitive antagonist of the NMDA receptor. It acts by binding inside the ion channel and preventing the influx of ions (similar to the Mg^{2+} block seen at resting membrane potentials).

In order to stop excitotoxicity in COS-7-cells transfected with GluN1 and GluN2B subunits DL-AP5 and MK801 were diluted to 10 mM and 100 nM respectively in H_2O and added to the cells approximately 6 hours after transfection (see 2.2.4).

2.5 Immunocytochemistry

Immunocytochemistry is used for; the detection of endogenous or exogenously expressed proteins and analysis of the subcellular localisation of endogenous and exogenous proteins in structurally intact cells. Adherent cells are grown on glass coverslips, fixed and permeabilised then incubated with protein specific antibodies and fluorescently labelled secondary antibodies. A fluorescent or confocal microscope can be used to visualise the cells and a number of imaging software can be used to analyse the cells.

2.5.1 Cell fixation and permeabilization

Cell fixation is used to preserve cells in a “life-like” state by stopping the activity of the cell and inhibiting autolysis. Cell membranes can be permeabilised by using detergents or solvents and is necessary for the detection of proteins which are not present at the cell surface.

For this study 4% paraformaldehyde (PFA – 2 g PFA in 50 mL PBS) was used. Cell medium was aspirated and 500µl of PFA was added to each well for 10minutes at room temperature. After which the cells were washed quickly 3x and permeabilized using 500 µl 0.2% triton-X for 10minutes. Cells were again washed 3x to remove excess detergent. Cells were immediately immunostained.

Primary Antibodies				
Antibody	**Species**	**Raised In**	**Source**	**Dilution**
NR1	Rat	Rabbit	Sigma	1/500
HA	16B12 Monoclonal	Mouse	Covance	1/2000
HA	Polyclonal	Goat	ABcam	1/500
FLAG	M2 Monoclonal	Mouse	Sigma	1/20000
V5	V5-10 Monoclonal	Mouse	Sigma	1/1000
ER	Calreticulin	Mouse	BD	1/500
ER	Calreticulin	Rabbit	ABCam	1/1000

Secondary Antibodies		
Alexa Fluour conjugated antibodies	**Source**	**Dilution**
Donkey anti-Mouse 350	Molecular probes	1/1000
Donkey anti-Mouse 488	Molecular probes	1/1000
Donkey anti-Rabbit 488	Molecular probes	1/1000
Donkey anti-Goat 594	Molecular probes	1/1000
Donkey anti-Mouse 594	Molecular probes	1/1000
Donkey anti-Rabbit 594	Molecular probes	1/1000
Chicken anti-Mouse 647	Molecular probes	1/1000
Chicken anti-Rabbit 647	Molecular probes	1/1000

Table 1

List of primary and secondary antibodies used, their source and the dilution in which they were used.

2.5.2 Immunostaining

2.5.2.1 Total staining

All immunostaining was performed at room temperature (unless otherwise stated). After fixation cells were blocked with 3% BSA/PBS for at least 30 minutes to block any non-specific binding sites. Both the primary and secondary antibodies were made up in 3 % BSA/PBS as detailed in Table 1. When 2 or more antibodies were used they had to be from different species (to avoid any cross reactivity of the secondary antibodies), and diluted together in blocking buffer. After blocking, the primary antibodies were added and the cells were left to incubate for 1 hour with gentle rocking. The cells were then washed 3x in PBS and an appropriate secondary antibody was added for 1 hour with gentle rocking. After the addition of the secondary antibody the dish was wrapped in tin-foil to avoid any photobleaching of the secondary antibodies. Cells were again washed 3x in PBS and mounted on glass microscope slides using mowiol mounting medium supplemented with 2 mg/mL DAPI when appropriate. The slides were wrapped in tin-foil and kept at 4 °C for at least 16 hours to allow the mowiol to set.

2.5.2.2 Surface labelling and receptor endocytosis

For some experiments it was necessary to label surface proteins before fixation. Cell media was aspirated and the cells were quickly washed with warm 3 % BSA/PBS. The appropriate primary antibodies were added and the cells were incubated with gentle agitation at 4 °C (to stop constitutive activity of the cell) for 1 hour. The cells were washed in PBS and incubated with the appropriate secondary antibody for 1 hour at 4 °C with gentle agitation and wrapped in tin-foil. Cells were then washed and fixed (as described in 2.5.1) or, for receptor internalization, fresh DMEM/FBS was added to the cells which were then returned to the incubator for 15 or 30 minutes before fixation.

2.5.3 Confocal microscopy

Laser scanning confocal microscopy allows the generation of highly detailed images from a single (or multiple) plane of focus of a sample. This is achieved by using a specific high-intensity monochromatic laser beam precisely focused to excite a

specific focal plane. Monochromatic mirrors and a pinhole aperture attached to a PMT exclude peripheral and out of focus light. Coupled to this florescent bleed through (when the emission spectrum of two fluorophores overlap) is reduced by sequential scanning of the sample. Individual lasers are used to excite individual fluorophores and the PMT aperture of the detector is finely set so only light emitted by one fluorophore is detected.

All images were captured using a Nikon A1R confocal microscope using its own software NIS elements 4.0.0.

2.5.4 Cell Surface Quantification

2.5.4.1 Surface expression quantification (no-internalisation)

For the quantification of surface expressed GluN2B when co-expressed with DISC1 or a DISC1 variant, COS-7 cells were transfected and stained as described in 2.2.4, 2.5.2.1, 2.5.2.2. Until completion of the analysis the operator was blinded to which DISC1 variant has been transfected to which set of cells. To ensure comparability of the images all images from a single experiment were taken on a Nikon A1R confocal microscope and all the images were taken at the same time using the same confocal settings.

Image analysis was performed using an I-Vision script developed in collaboration with Paul Perry, IGMM University of Edinburgh. For each image the four colour image was split into its individual channels, removing one and merging them into a 3 colour image and converting the image into TIFF format. These 3 colour images are loaded into I-Vision where the channel with the surface expressed GluN2B was loaded and the user is prompted to encircle the cell of interest creating a region of interest (ROI). The script then assigns a mask around the cell and measures the mean pixel intensity under this mask. A second line is drawn around the mask and imposed onto the image showing the DISC1 signal. Using the third image a mask is drawn around the nucleus and is also imposed onto the image with the DISC1 signal. The mean pixel intensity between the two masks is taken, thus generating the DISC1 expression level. The nucleus is omitted as some of the DISC1 variants are

not expressed in the nucleus and would therefore give an overall lower signal value. A table is produced recording this measurement which is saved for statistical analysis.

2.5.4.2 Incubated surface labelled quantification

To quantify internalised protein, the image was opened in ImageJ and a region of interest was drawn around the cell. The Pearsons co-efficient was generated using the co-localisation threshold plugin which determines the Pearsons coefficient of two specified channels. Pearsons coefficient is a measure of the overlap between two separate objects, in cell biology this usually requires two objects to be lablled (in this case with fluorophores) and if the two objects overlap sufficiently then they are deemed to be close enough to interact. The Pearsons coefficient measures the amount the signal from each protein and calculates how much of the signal overlaps with the other signal and generates a value from -1 to +1, with -1 = total negative correlation, 0 = no correlation and +1 is total positive correlation. So the closer the Pearsons coefficient is to +1 then the more chance of the two proteins of interest have of being close enough to interact.(Adler and Parmryd, Dunn et al.).

The plugin can be found at: http://www.uhnresearch.ca/facilities/wcif/fdownload.html

2.6 Molecular biology methods

2.6.1 Plasmids

Plasmids used during the course of this PhD are described in Table 2. All were gifts from the specified person and were transformed and purified as appropriate as described in 2.6.2 and 2.6.3.

Plasmid Name	Encoded protein	Tag	Source
pCB6/NR1	Rat GluN1	None	William Green[1]
pCB6/HA-GluN2B	Rat GluN2B	HA N-term	William Green[1]
pcDNA/TO-FLAG DISC1	Human DISC1	FLAG N-term	Elise Malavasi[2]
pcDNA/TO-FLAG DISC1-607F	Human DISC1	FLAG N-term	Elise Malavasi[2]
pcDNA/TO-FLAG DISC1-37W	Human DISC1	FLAG N-term	Elise Malavasi[2]
pcDNA/TO-FLAG DISC1-704C	Human DISC1	FLAG N-term	Elise Malavasi[2]
pcDNA/TO FLAG-TRAK1	Human TRAK1	FLAG N-term	Fumiaki Ogawa[2]
pcDNA/TO FLAG-TRAK1-H678R	Human TRAK1	FLAG N-term	Fumiaki Ogawa[2]
pDEST40NDE1	Human NDE1	V5 C-term	Kirsty Millar[2]
pDEST40NDE1-T131A	Human NDE1	V5 C-term	Nick Bradshaw[2]
pDEST40NDE1-T131E	Human NDE1	V5 C-term	Nick Bradshaw[2]
pmCherry-C1/RAB5	RAB5	mCherry N-term	Vilma Martins[3]
pmCherry-C1/RAB7	RAB7	mCherry N-term	Vilma Martins[3]
pMAX-GFP	GFP	None	Fumiaki Ogawa[2]
PCMV/Globin	Human Globin	None	Giles Hardingam[2]
FLAG-pcDNA 4TO	Empty Vector	HA N-term	Elise Malavasi[2]
pDEST40	Empty Vector	V5 C-term	Kirsty Millar[2]

Table 2

A list of all plasmids used in this thesis, their source and any tag the protein will possess. [1] University of Chicago. [2]University of Edinburgh. [3]National Institute for Translational Neuroscience and National Institute of Oncogenetics, São Paulo, Brazil.

2.6.2 Transformation

Transformation of E. coli allows for the amplification of existing plasmids. DH5α-competent cells (Invitrogen) were used to amplify existing plasmids.

An aliquot of cells were thawed on ice and the cell suspension was transferred to a sterile tube. 1-5 µl of 10mg DNA was added to the cell suspension and was gently mixed by swirling the pipette tip and incubated on ice for 30minutes. The cells were then heat shocked at 42 °C followed by an incubation on ice for 2 minutes. 200 µl of pre-warmed S.O.C (Invitrogen) was added and the cells were incubated at 37 °C shaking at 220 rpm for 1-2 hours. 100 µl of the suspension was then evenly spread over an agar plate inoculated with the appropriate antibiotic. The plates were left at 37 °C for 16-22 hours or when bacterial colonies could be seen.

2.6.3 Purification of plasmids from E.Coli

In order to purify DNA from transformed cultures commercial kits from Qiagen were used. Individual colonies were picked from the agar plate using a sterile pipette tip which was placed into 5 mL lysogeny broth (LB) inoculated with the appropriate antibiotic for 4-8 hours while shaking at 250rpm. 1.5 mL of the culture was spin at 13,000rpm for 1 minute, the supernatant removed and another 1.5 mL was added and spun again. The supernatant was removed and the pellet was left for 1-2 minutes to dry. Plasmid DNA was purified from the pellet using a Qiagen spin mini-prep kit according to the manufacturers' directions. To make larger volume and concentration of plasmid DNA, colonies were grown in 5 mL LB supplemented with the appropriate antibiotic for 6-8 hours shaking at 220rpm at 37 °C and 1 mL was added to 400 mL LB supplemented with the relevant antibiotic and left shaking at 37 °C overnight. The culture was spun at 6000 g for 15minutes at 4 °C. The supernatant was discarded and plasmid DNA was extracted using a Qiagen Maxi-prep kit using the manufacturers' guidelines.

2.6.4 Measuring DNA concentration

To measure the concentration of newly purified plasmid DNA a spectrophotometer was used. Purified plasmid was diluted to 1 in 20 and after calibrating the machine

with water, 1 mL of sample was analysed and the wavelength noted down. The concentration of DNA was calculated using the following equation:

$$[DNA] = A260 \times dilution\ of\ plasmid \times 0.05$$

2.6.5 DNA sequencing

Sequencing was used to verify protein expression plasmids, specifically the DISC1, DISC1 variants, NDE1 and phosphomutant NDE1 constructs. To do this Big Dye terminator (ABI) kit was used. In brief the plasmid DNA was diluted to 300 ng and a reaction mix was assembled:

1.5 µl 5x Big Dye Buffer

1.0 µl 3.2pMol/ µl sequence primer

1.0 µl Big Dye enzyme

300 ng DNA

H_2O to 10 µl

This was assembled in a 500 µl eppendorf tube and ran on a PTC-225 deltimhouse cycle using the following programme:

1. 96 °C 1min
2. 96 °C 10sec
3. 50 °C 5sec
4. 60 °C 4min
5. Repeat 2-4 24 times
6. 4 °C holding temp

After the sample had cooled to 4 °C the samples were removed and 2.5 µl 125mM EDTA, and 30 µl 100 % ethanol added. The samples were briefly vortexed to mix and left for 15 minutes at room temperature. The samples were spun at 1300 rpm for 20 minutes at 4 °C and supernatant was removed. 30 µl 70 % ethanol was added

and the samples were spun for 5 minutes at 13,000 rpm at 4 °C. The supernatant was removes and the samples were left to dry for 5 minutes and stored at -20 °C. The nucleic acid sequence was determined by the sequencing service at the IGMM. DNA sequences were viewed and analysed using Finch TV version 1.4.0 (Geospiza).

2.7 Electrophysiology

Dissection and culturing of neurons used for electrophysiology were kindly performed by the Hardingham lab (McKay et al., 2012). Neurons were cultured on coverslips and transfected with PMAX GFP + DISC1 L607F-DISC1 or globin as described in 2.2.3 at DIV 7 or 8 and recorded from on DIV 9 or 10.

Coverslips were transferred to a recoding chamber perfused with the following external recording solution:

NaCl : 150 mM

KCL: 2.8 mM

HEPES: 10 mM

$CaCl_2$: 2 mM

$MgCl_2$: 1 mM

Glucose: 10 mM

Glycine: 100 µm

Tetrodotoxin: 100 nM

pH to 7.3 – 7.35 with NaOH

osmolarity between 300 – 350 osm

Patch pipettes were made from thick walled borosilicate glass (Harvard) and filled with internal solution consisting of:

K-Gluconate: 141 mM

NaCl: 2.5 mM

HEPES: 10 mM

EGTA: 11 mM

Adjust to pH 7.3 with KOH

Electrode tips were fire polished for a final resistance ranging between 3 and 7 MΩ. Currents were recorded at room temperature (21±2 °C) and all recordings were digitally stored on MATLAB. Neurons were voltage clamped at -65 mV and recordings were repeated if the holding current changed by more than ±100 pA during the recording or if the series resistance drifted by more than 20 % of its initial value.

3 Selection of proteins and variants for study

3.1 Aims

To identify proteins in the DISC1 complex that have potential to participate in NMDA receptor trafficking for testing in the surface expression assay described in chapter 4.

3.2 Introduction

DISC1 is known to play a role in trafficking events within the cell, through an involvement in mitochondrial trafficking and synaptic vesicle trafficking, as discussed in chapter 1. These observations point towards trafficking disruptions as potentially important in searching for the disease mechanisms that underlie mental illness. Because DISC1 is already known to be involved in trafficking of mitochondria and synaptic vesicles (Atkin et al., 2010, Ogawa et al., 2013, Flores Iii et al., 2011, Maher and LoTurco, 2012), I hypothesised that it has a general role in trafficking that extends to NMDA receptors. I therefore set out to investigate the role of DISC1 in NMDA receptor trafficking from the endoplasmic reticulum onwards, and to determine whether DISC1 sequence variants have any effect upon this process.

A number of DISC1 sequence variants have been found to cause changes in DISC1 function, and I chose to investigate three of these; R37W, L607F and S704C. The minor allele of the common variant L607F has been shown to cause a number of deficiencies when compared to wild-type DISC1 function (Brauns et al., 2011, Malavasi et al., 2013, Soares et al., 2011). Of particular relevance to this thesis, unlike wild-type DISC1, DISC1-607F fails to rescue mitochondrial trafficking deficits caused by DISC1 knock down (Atkin et al., 2010), thus it adversely influences the role of DISC1 in trafficking.

The ultra-rare DISC1 variant 37W has also been shown to influence mitochondrial trafficking, by inhibiting DISC1's ability to promote anterograde mitochondrial movement (Ogawa et al., 2013). This effect may be related to increased DISC1 association with the trafficking molecule TRAK1 due to the 37W variant (Ogawa et al., 2013). Preliminary evidence indicates that it may also impact upon DISC1

association with the NMDA receptor subunit GluN1 and upon GluN1 phosphorylation (K. Millar, unpublished).

In contrast there is currently no evidence that the S704C variant influences intracellular trafficking. The minor allele does, however, influence DISC1 interaction with the trafficking molecules NDE1 and NDEL1 (Leliveld et al., 2008, Leliveld et al., 2009), suggesting that it may affect dynein-mediated transport.

In addition to investigating a potential role for DISC1 in NMDA receptor trafficking, and any effects of the DISC1 sequence variants 37W, 607F and 704C, I also set out to investigate whether any DISC1-associated proteins may also be involved in the process. Because of their known involvement in trafficking events, I selected NDE1 and TRAK1 for study. Moreover, endogenous NDE1 has been shown to associate with TRAK1 independently of DISC1 in HEK293 cells, SH-SY5Y cells and in mouse brain (F. Ogawa & K. Millar, unpublished), indicating that they likely function together in regulating intracellular transport. In addition, a TRAK1 sequence variant, 678R, has been identified in a single schizophrenic patient so I chose to test whether this putatively causal variant affects any potential role of TRAK1 in NMDA receptor trafficking.

In this chapter I describe preliminary investigation of these proteins and sequence variants to test their potential to be involved in NMDA receptor trafficking.

3.3 DISC1 localises to the endoplasmic reticulum in the presence of NMDA receptors

Although DISC1 is known to localise to multiple subcellular compartments including the nucleus, centrosome, mitochondria and Golgi apparatus, it has not been shown to be targeted to the endoplasmic reticulum, the site of NMDA receptor synthesis and assembly. I wanted to determine which stages of NMDA receptor trafficking might be influenced by DISC1, therefore I first determined whether DISC1 is ER-associated. COS7 cells were transfected with FLAG-DISC1 and co-stained with anti-calreticulin to stain the ER (Figure 3.1 A-C). There was no obvious co-localisation between DISC1 and the ER marker, indicating that DISC1 is likely not present there in significant quantities when overexpressed in COS7 cells. However, because DISC1 complexes with NMDA receptors (S.Mackie unpublished), I wanted to determine whether the presence of the receptor affects DISC1 subcellular distribution. DISC1, GluN1 and GluN2B were co-expressed in COS7 cells, the cells were fixed and stained for GluN1, GluN2B DISC1 and the ER. Under these circumstances DISC1 apparently co-localises with the ER marker calreticulin to approximately the same extent as GluN1 or GluN2B, thus all three proteins may associate at the ER (Figure 3.1 D-K). There is potential therefore, for DISC1 to associate with NMDA receptors from the ER onwards during the forward trafficking process.

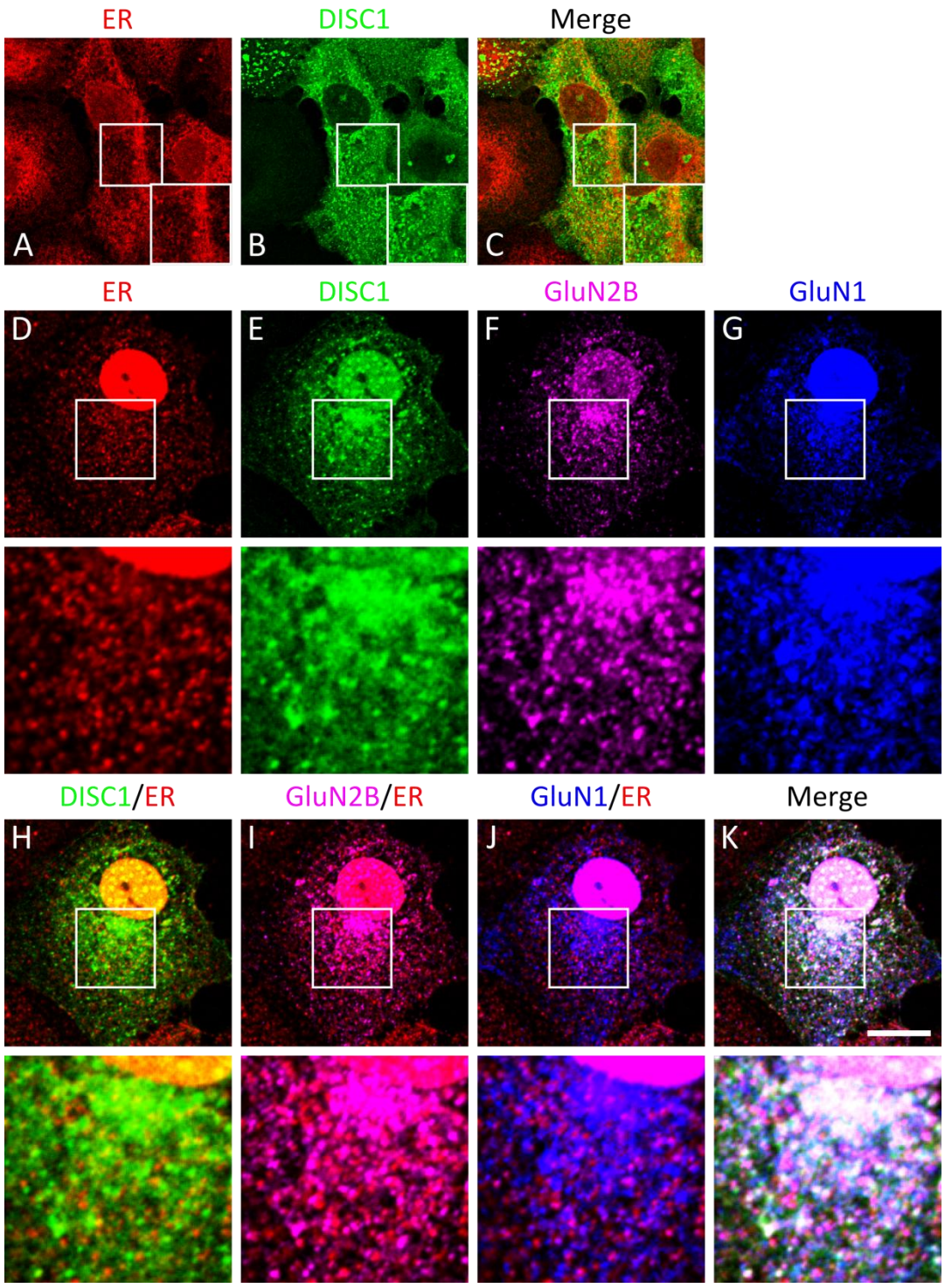

Figure 3.1 DISC1 co-localises with GluN1 and GluN2B within the ER and at the surface of COS7 cells.

COS7 cells transfected with FLAG-DISC1 (A) and co-stained with anti-calreticulin to stain the ER (B) show no DISC1/ER co-localisation (C). Cells co-transfected with FLAG-DISC1 (E), HA-GluN2B (F) and GluN1 (G) and co-stained with anti-calreticulin (D) show co-localisation between DISC1/ER (H) GluN2B/ER (I) and GluN1/ER (K) indicating that the presence of GluN1 and GluN2B may redistribute DISC1 to the ER. n = 3 Scale bar 20 µm, white boxes are enlarged below. GluN2B pseudocoloured magenta using ImageJ.

3.4 TRAK1 co-localises robustly with NMDA receptors

Overexpression of TRAK1 in COS7 cells leads to its accumulation at mitochondria and causes mitochondrial clustering (Ogawa et al., 2013) Figure 3.2 A-C). I could find no evidence of TRAK1 ER-localisation in COS7 cells transfected with FLAG-TRAK1 and co-stained with anti-FLAG and anti-calreticulin antibodies (Figure 3.2 D-F). FLAG-TRAK1 carrying the 678R mutation identified in a schizophrenia patient localises similarly to wild-type TRAK1 (Figure 3.2 G-L).

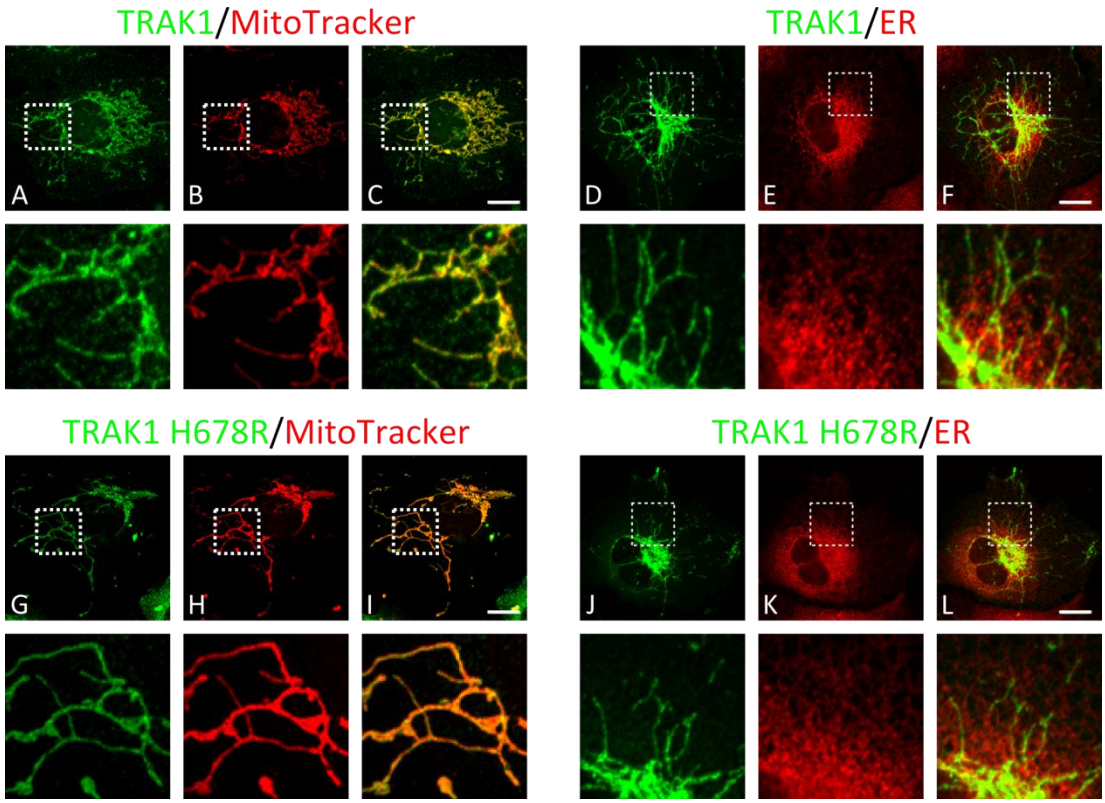

Figure 3.2 TRAK1 and TRAK1-678R do not co-localise with the ER.

COS7 cells transfected with TRAK1 (A) and co-stained with MitoTracker (B) show significant co-localisation (C). TRAK1 (D) does not co-localise with calreticulin (ER marker E and F) indicating little or no ER localisation. COS7 cells transfected with TRAK1-678R (G) co-localise with MitoTracker (H and I). Like TRAK1, TRAK1-678R (J) does not co-localise with the ER marker calreticulin (K and L). White boxes are enlarged below. n = 3 Scale bar 20 µm.

Next, although TRAK1 is exclusively mitochondrial when expressed in COS7 cells, and mitochondria are not known to be a site of NMDA receptor expression, I examined co-localisation between TRAK1 and GluN1 or GluN2B in COS7 cells.

Co-expression of HA-GluN1 and FLAG-TRAK1 did not result in any change in subcellular localisation for either protein. As expected, HA-GluN1 localises to the ER in the presence or absence of FLAG-TRAK1 (Figure 3.3 A-D). Similarly, FLAG-TRAK1 localises to mitochondria irrespective of GluN1 (Figure 3.3 A-D). I could therefore find no evidence of an association between TRAK1 and GluN1, as expected. Interestingly, however, this was not the case for TRAK1 and GluN2B. FLAG-TRAK1 and HA-GluN2B co-expression in COS7 cells substantially altered the distribution of GluN2B and resulted in significant co-localisation of the two proteins (Figure 3.3 E-H). This dramatic co-localisation suggested a strong association between the two proteins which was confirmed by co-immunoprecipitation of overexpressed TRAK1 and GluN2B (S. Mackie & K. Millar, unpublished) and by co-immunoprecipitation of endogenous TRAK1 and GluN2B from mouse brain synaptosomes (F. Ogawa & K. Millar, unpublished). Unexpectedly therefore, there is a robust association between TRAK1 and the NMDA receptor GluN2B subunit.

Having established that TRAK1 co-localises robustly with GluN2B, but not GluN1, I next determined whether assembled GluN1/GluN2B-containing NMDA receptors can associate with TRAK1. Importantly, co-expression of HA-GluN2B, GluN1 and FLAG-TRAK1 in COS7 cells resulted in co-localisation of all three proteins with the mitochondrial marker MitoTracker (Figure 3.3 I-M). The TRAK1/GluN2B association is therefore maintained when GluN2B is assembled with GluN1, and GluN1 appears to be recruited to mitochondria via the association of its binding partner GluN2B with TRAK1. It is likely that the mitochondrial NMDA receptor association in COS7 cells is an artefact of protein overexpression because these receptors are not known to localise to mitochondria, but that said, my data, and the data from other members of the group, do indicate that TRAK1 may associate with assembled NMDA receptors.

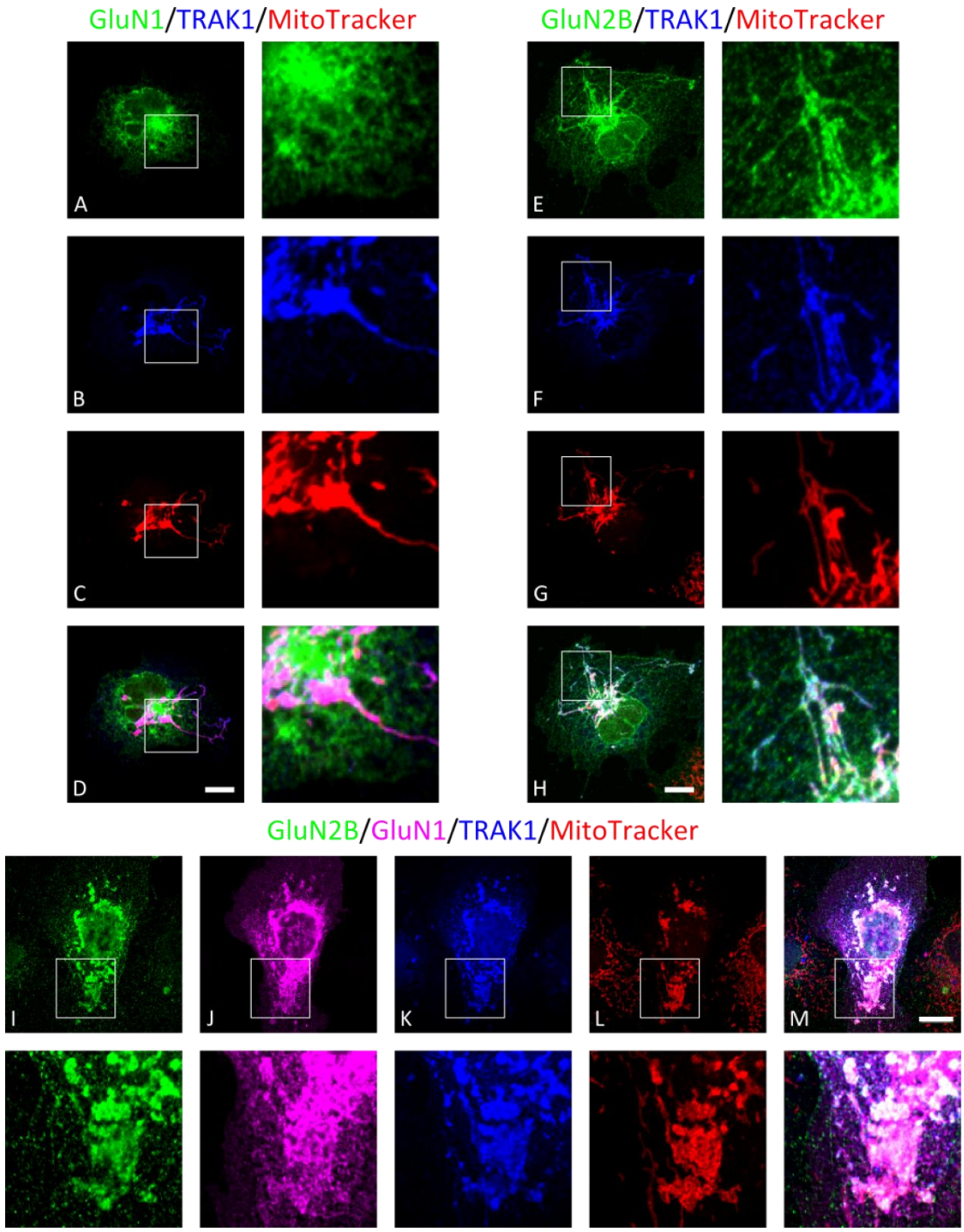

Figure 3.3. TRAK1 co-localises with GluN2B and assembled NMDA receptors in COS7 cells.

COS7 cells overexpressing GluN1 (A) and TRAK1 (B) were co-stained with MitoTracker (C). There is co-localisation between TRAK1 and MitoTracker but not between GluN1 and TRAK1 or MitoTracker (D). COS7 cells overexpressing GluN2B (E) and TRAK1 (F) were co-stained with MitoTracker (G) which leads to co-localisation between all three signals (H). Co-expression of GluN2B (I), GluN1 (J) and TRAK1 (K) leads to co-localisation (M) of all three signals with MitoTracker (L) indicating recruitment to the mitochondria. White boxes are enlarged beside (A-H) and below (I-M). n = 3, Scale 20 µm. GluN1 image in bottom panel (J) pseudocoloured magenta using ImageJ.

To confirm the TRAK1/GluN2B co-immunoprecipitation data, I then examined the association between TRAK1 and NMDA receptor subunits in neurons. FLAG-TRAK1, GluN1 and HA-GluN2B were transfected into hippocampal neurons at DIV 17 and processed at DIV18. Overexpression of these proteins leads to expression in both the axons and the dendrites (Figure 3.4 A-C). TRAK1 and GluN2B co-localise in dendritic spines and along dendritic shafts, with similar co-localisation detectable between GluN1 and TRAK1 (Figure 3.4 E). This provides further support for an association between NMDA receptors and the trafficking molecule TRAK1, and supports my hypothesis that the DISC1 complex regulates NMDA receptor trafficking.

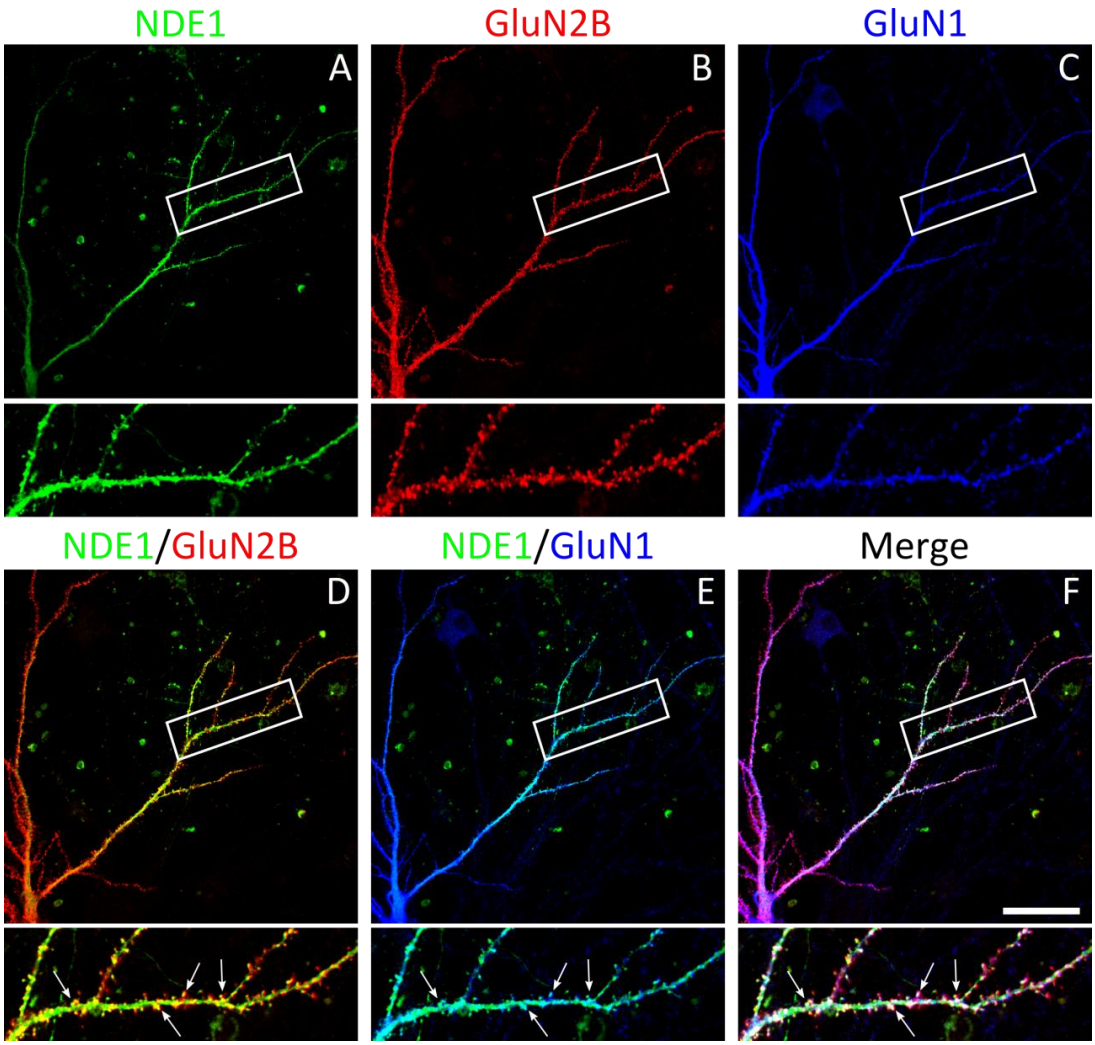

Figure 3.4 TRAK1 co-localises with GluN1 and GluN2B in neurons.

DIV17 C57 hippocampal neurons were transfected with GluN1, HA-GluN2B and FLAG-TRAK1. At DIV18 the neurons were stained for TRAK1 (A) which is present throughout the dendrites and axon. GluN2B shows a largely similar staining pattern (B) as does GluN1 (C). A merge of TRAK1 and GluN2B (D) shows significant co-localisation of the two signals, White boxes are enlarged below and white arrows indicate spines expressing both proteins. Similarly, merging TRAK1 and the GluN1 channels results in significant co-localisation of the two signals. This indicates a possible association of TRAK1 and NMDA receptors in spines in hippocampal neurons. n = 3, Scale bar 20 µm.

3.5 NDE1 may associate with NMDA receptors

I set out to examine whether NDE1 may associate with NMDA receptors by transfecting COS7 cells with GluN1 and V5-NDE1 (Figure 3.5 A-C). Both proteins were expressed in a similar pattern, but it was difficult to distinguish clear co-localisation. Cells were also transfected with HA-GluN2B plus V5-NDE1, with similar results (Figure 3.5 D-F). Next, COS7 cells were co-transfected with GluN1, HA-GluN2B and V5-NDE1, and stained for all three proteins (Figure 3.5 G-I). Under these conditions there was clear co-localisation between NDE1 and a subset of the GluN2B and GluN1 (Figure 3.5 J), indicating a possible association between NDE1 and assembled NMDA receptors.

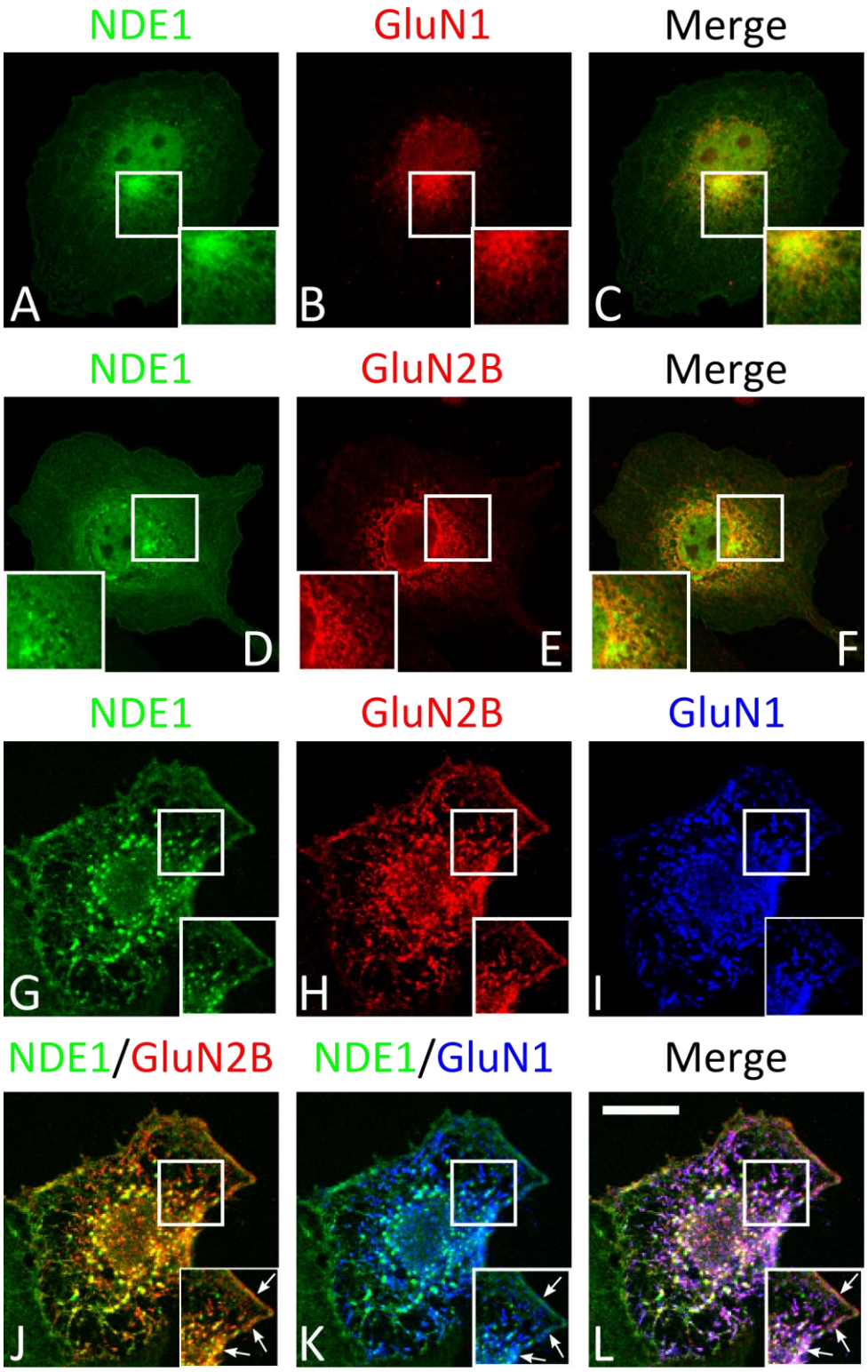

Figure 3.5 NDE1 may associate with assembled NMDA receptors.

COS7 cells transfected with V5-NDE1 (A) and GluN1 (B) show little obvious NDE1/GluN1 co-localisation (C). Similarly cells expressing V5-NDE1 (D) and HA-GluN2B (E) show little obvious co-localisation (F). Cells expressing NDE1 (G), GluN2B (H) and GluN1 (I) show co-localisation between within the cytoplasm and at the surface of the cell, as indicated by white arrows. n = 3, Scale bar 20 µm, white boxes are enlarged in the corner of the image.

I also examined NDE1 co-localisation with NMDA receptor subunits in primary cultured mouse hippocampal neurons. Mouse hippocampal neurons were transfected at DIV17 with GluN1, HA-GluN2B and V5-NDE1, and stained at DIV 18 for GluN1, HA and V5 (Figure 3.6). All three proteins were expressed throughout the soma, dendrites and axon, with clear co-localisation within dendrites and dendritic spines.

Taken together these data indicate that NDE1 and NMDA receptors may associate in neurons, therefore there is potential for NDE1 to have a role in NMDA receptor trafficking.

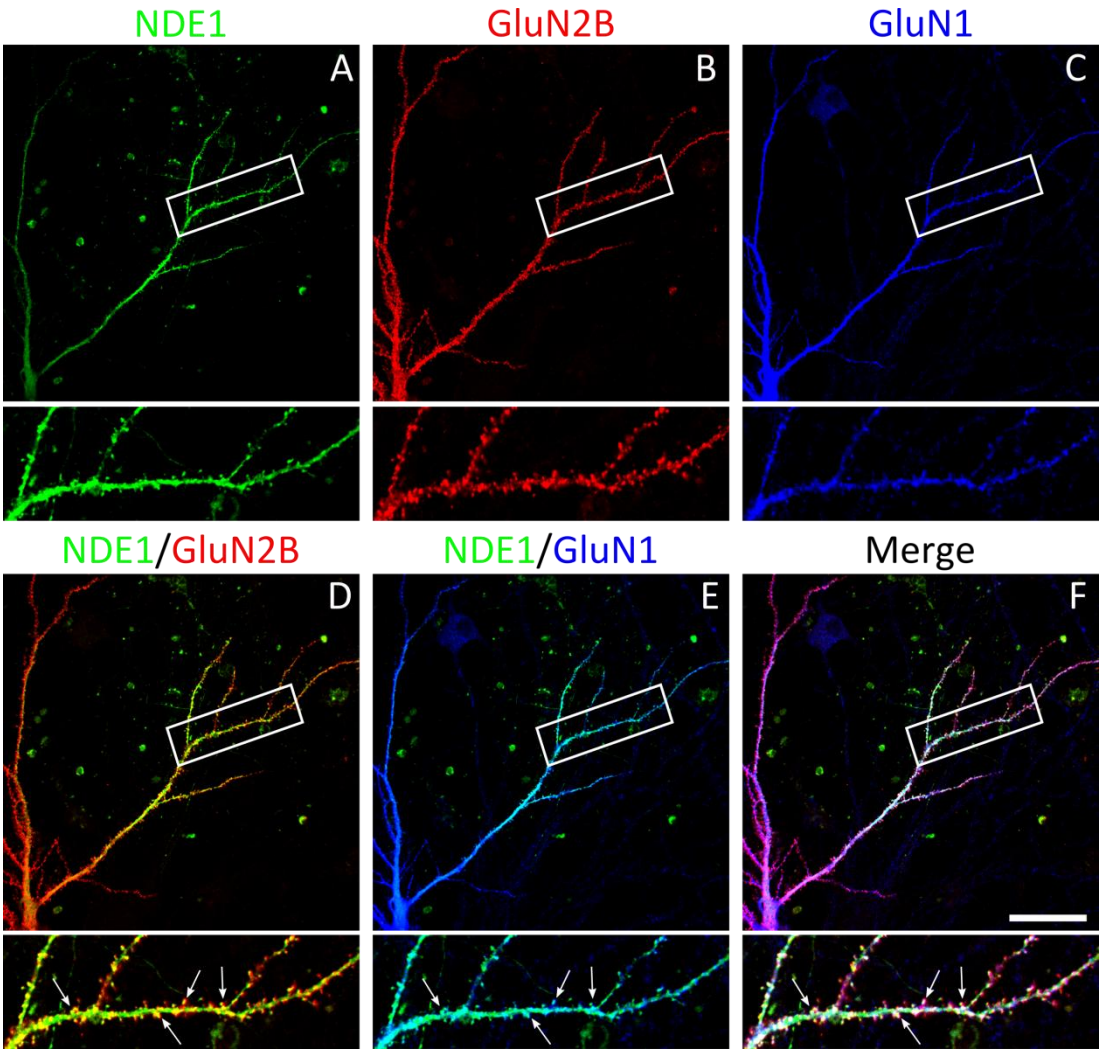

Figure 3.6 NDE1 co-localises with GluN1 and GluN2B in neurons

DIV18 C57BL/6 hippocampal neurons were transfected with GluN1, HA-GLuN2B and V5-NDE1. The neurons were stained for NDE1 (A) which is and is present throughout the dendrites and axon, GluN2B (B) and GluN1 (C). A merge of NDE1 and GluN2B signal (D) shows clear co-localisation. White boxes are enlarged below, and white arrows indicate spines where both proteins co-localise. Similarly, merging NDE1 and GluN1 signals revealed significant co-localisation. n = 3, Scale bar 20 µm.

3.6 Discussion

In this chapter I have provided suggestive evidence that DISC1 may associate with both GluN1 and GluN2B subunits of the NDMA receptor within the ER when co-expressed in COS7 cells. Moreover, DISC1 has also been shown to localise to the Golgi (Kuroda et al., 2011, Lepagnol-Bestel et al., 2013). Coupled with the data showing that DISC1 binds to GluN1 subunits of the NMDA receptor (S. Mackie & K. Millar, unpublished) and the fact that DISC1 is known to be involved in intracellular trafficking (Shinoda et al., 2007, Ozeki et al., 2003, Morris et al., 2003, Ogawa et al., 2005, Brandon et al., 2004), there is clearly potential for involvement of DISC1 in trafficking of the NMDA receptor. DISC1 could act at many levels. 1) DISC1 regulates PKA signalling via modulation of PDE4 cAMP hydrolysis activity (Millar et al., 2005b, Bradshaw et al., 2008) and could therefore potentially regulate phosphorylation of sites that influence receptor ER-retention and forward trafficking from the ER (Scott et al., 2001, Scott et al., 2003). 2) DISC1 could directly participate in receptor trafficking via association with TRAK1 and NDE1, and its reported association with the molecular motors dynein and kinesin (Taya et al., 2007, Kamiya et al., 2005). 3) DISC1 may be involved in NMDA receptor recycling at the cell surface as it is has been reported to co-localise with RAB11, a marker of the recycling endosome (Lepagnol-Bestel et al., 2013).

I also identified a novel robust association between GluN2B and TRAK1. The strong association between these two proteins in mouse brain, and my data indicating that TRAK1 also associates with assembled NMDA receptors, is indicative of a role for TRAK1 in NMDA receptor trafficking. Consistent with this, in neurons TRAK1 is known to traffick $GABA_A$ receptors and endosomes, as well as mitochondria (Webber et al., 2008, Brickley et al., 2005, Ogawa et al., 2013, Brickley et al., Brickley and Stephenson, 2011), therefore it is possible that NMDA receptors could

be an additional cargo. Indeed in neurons I observed co-localisation between TRAK1 and NMDA receptors.

In COS7 cells endogenous TRAK1 has been shown to co-localise with an ER marker (Webber et al., 2008). Because TRAK1 is a kinesin adaptor molecule it could therefore potentially be involved in NMDA receptor forward trafficking from the ER to the surface of the cell. Furthermore endogenous TRAK1 has been shown to associate with the early endosome (Webber et al., 2008), and with dynein (van Spronsen et al., 2013). Consequently, because the NMDA receptor is a highly dynamic receptor which is constantly endocytosed, TRAK1 could conceivably be involved with this process as well.

In COS7 cells the TRAK1/NMDA receptor association apparently occurs exclusively at mitochondria. It is likely that this is an artefact due to the strong targeting of TRAK1 to mitochondria in COS7 cells, and it is improbable that NMDA receptors are genuinely targeted to mitochondria via TRAK1 association *in vivo*. Indeed, as already discussed, TRAK1 is not exclusively mitochondrial in neurons. It is an interesting possibility however, that NMDA receptors and mitochondria could be co-transported within neurons, but this is not something I was able to investigate in this thesis.

I additionally obtained evidence that the dynein regulator NDE1 may associate with GluN1 and GluN2B subunits, although further work, such as co-immunoprecipitation experiments, is required to confirm this possibility. Previous studies showed that NDE1 localises to the glutamergic synapse (Bradshaw et al., 2008). Here I showed co-localisation of NDE1 and GluN1 and GluN2B within the spines of dendrites, which is consistent with a role for NDE1 in trafficking NMDA receptors to the synapse. Given what is known about NDE1 function within the cell, it could regulate NMDA receptor trafficking at two stages. 1) NDE1 is known to be present at the ER (Lam et al., 2009) where the NMDA receptor is retained until correctly assembled, before being trafficked to the surface of the cell. As NDE1 plays a critical role in intracellular trafficking it could be involved in trafficking of the

receptor from the surface 2) At the cell surface, NDE1 could regulate NMDA receptor endocytosis via its role in modulation of dynein and of endosomes (Lam et al., 2009).

To summarise, we now know that DISC1, and TRAK1 associate with NMDA receptors, and that NDE1 associates robustly with DISC1 and NDE1, and may associate with NMDA receptors. All three molecules regulate intracellular trafficking events and the role of DISC1 in trafficking is influenced by DISC1 amino acid variants. In the next chapters I will describe my investigation into the role of DISC1, TRAK1 and NDE1 in NMDA receptor trafficking.

4 Development and optimisation of the surface expression assay and surface quantification programme

4.1 Introduction

The GluN1 and GluN2 subunits of the NMDA receptor co-translationally assemble in the ER to form functional channels (McIlhinney et al., 1998, Monyer et al., 1992, Ozawa et al., 1998). When expressed individually, GluN1 and GluN2B subunits are retained in the ER (McIlhinney et al., 1998, McIlhinney et al., 1996). A number of studies of mammalian cell lines transfected (either transiently or permanently) with GluN1 subunits indicate that, when expressed alone GluN1 does not give rise to a glycine-glutamate responsive channel but instead requires the presence of the GluN2 subunit in order to form such a channel. (Cik et al., 1993, Varney et al., 1996, Grimwood et al., 1995). Co-precipitation of *in vitro* synthesised GluN1 and GluN2B can be achieved when both subunits are expressed together, indicating that these subunits bind to one another (Mcilhinney et al 98). Interestingly, transfecting COS7 cells with GluN1 and GluN2B separately (with subsequent mixing) does not result in co-precipitation, suggesting that their association relies on co-synthesis (Mcilhinney et al 98). Fukaya et al (Fukaya et al., 2003) showed that GluN2B subunits are retained in the ER in GluN1 knock-out mice (targeted knock-out to the CA1 pyramidal cells of the hippocampus) further demonstrating that both subunits are required to form a functional channel.

As described in the introductory chapter studies of NMDA receptor trafficking indicate tight regulation of GluN1 and GluN2B subunit expression. ER retention occurs until the full NMDA receptor is formed, it is then forward trafficked to the surface. In this chapter I will describe optimisation of an assay for specifically detecting the surface expression of NMDA receptors, and use of this assay to compare how different proteins of interest may affect the surface expression of NMDA receptors. I will then describe generation of a script using image analysis software which allows me to quantify the signal from the surface NMDA receptors, thus generating a robust way of analysing the data.

4.2 Methods optimisation

4.2.1 GluN1 and GluN2B localise to the ER as expected

As described previously, GluN1 and GluN2B subunits of the NMDA receptor are retained in the ER until co-expressed. To ensure the GluN1 and HA-GluN2B constructs I will be using express the protein correctly, a number of co-localisation studies were carried out. COS7 cells were transfected separately with the GluN1 and HA-GluN2B subunits and co-stained with an antibody specific for the ER (calreticulin). From the literature the localisation of these proteins should be exclusively within the ER. Figure 3.1 shows clear and extensive co-localisation between GluN1 and the ER (Figure 3.1A-C). Similarly this was observed for cells expressing GluN2B and co-stained with the calreticulin (Figure 3.1D-F). This was observed in 100% of the cells transfected. When the subunits are co-expressed there is a much more even expression throughout the cell than compared to individually expressed subunits. Also there is a much stronger presence at the surface of the cell which is very apparent in the enlarged images (Figure 3.1G-I).

These observations follow the published literature, thus the plasmids I will be using express GluN1 and GluN2B subunits that are retained within the ER and expressed as they should be.

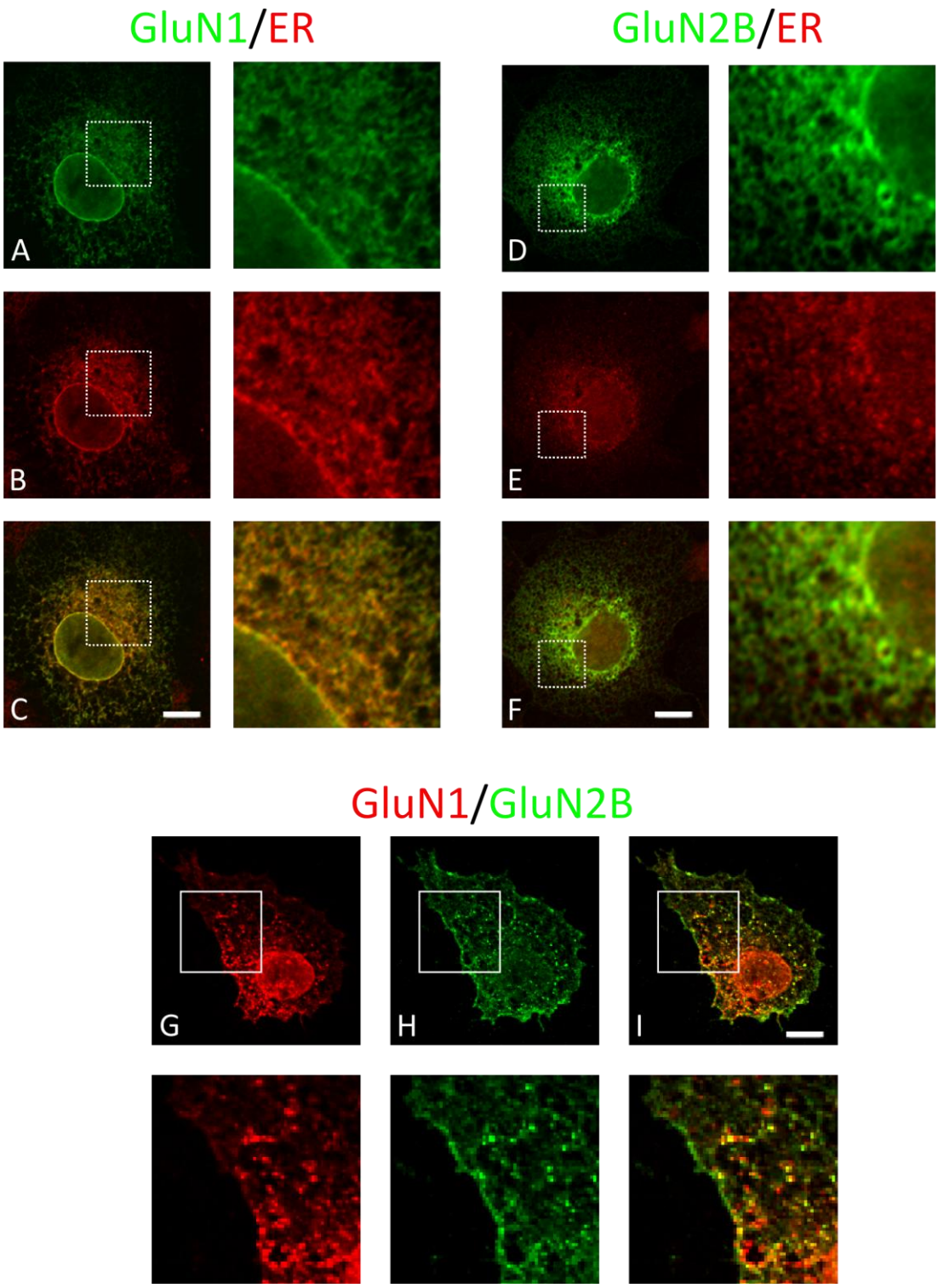

Figure 4.1 Individually expressed GluN1 and GluN2B subunits co-localise with the ER. Co-expression leads to additional expression at the surface.

Representative images of COS7 cells overexpressing GluN1 or HA-GluN2B and co-stained with calreticulin (ER). GluN1 shows significant co-localisation with the ER (A, B, merged in C). HA-GluN2B also shows significant overlap with the ER (D, E, merged in F). Co-expression of GluN1 and GluN2B leads to surface expression (G, H, merged in I). Boxed areas are shown enlarged. n = 3, Scale bar 20 µm.

4.2.2 Optimisation of the surface expression assay

In order to determine the effect of DISC1 and its binding partners on NMDA receptor trafficking I optimised a trafficking assay in COS7 cells. A study by Horack et al showed specific surface staining of NMDA receptors in COS7 cells. The group co-transfected the cells with GluN1 and GFP-GluN2B. As the GFP- tag is located on the extracellular N-terminus they could specifically label the surface NMDA receptors using an anti-GFP antibody. This incubation was carried out before fixation, so there is no labelling of intracellular protein, and at 4 °C to inhibit any constitutive internalization of the NMDA receptor (Horak et al., 2008).

As I am interested in determining the effects of DISC1 on NMDA receptor surface expression I transfected COS7 cells with; GluN1 and HA-GluN2B or GluN1, HA-GluN2B plus FLAG-DISC1. To examine surface NMDA receptors via visualisation of GluN2B, transfected cells were labelled live with anti-HA at 4 °C, followed by an appropriate secondary antibody to specifically detect GluN2B expressed at the cell surface. The cells were next fixed, permeabilized and stained with anti–HA and anti-GluN1 to detect the total GluN2B and GluN1 populations, respectively. Figure 4.2 (A-D) shows the resulting staining pattern, the surface GluN2B staining shows a punctate staining pattern, which is in line with that published in the literature. Also the total staining for GluN1 and HA-GluN2B gives a clear signal and clearly shows adequate amounts of subunit are present within the cell. Similarly, using an antibody specific for the N-terminus of the GluN1 subunit, surface staining was carried out on live cells in the same way as for HA-GluN2B. This produced a similar pattern of expression as the HA surface labelling (Figure 4.2 E-H). In cells expressing HA-GluN2B, GluN1 and FLAG-DISC1 the HA-GluN2B surface staining is again punctate in appearance, and the total staining patterns of GluN1 and HA-GluN2B indicate they are present at adequate concentrations (Figure 1.2 I-K). Finally the staining for DISC1 (Figure 1.2 L) appears punctate as has been described in several

publications, and exhibits extensive overlap with surface-expressed and intracellular NMDA receptor subunits.

This assay clearly gives specific staining for; surface expressed NMDA receptors, the whole population of GluN subunits and of an additional protein, in this case DISC1. Therefore the assay is suitable for determining whether DISC1, or another protein, can influence the surface expression of NMDA receptors in COS7 cells.

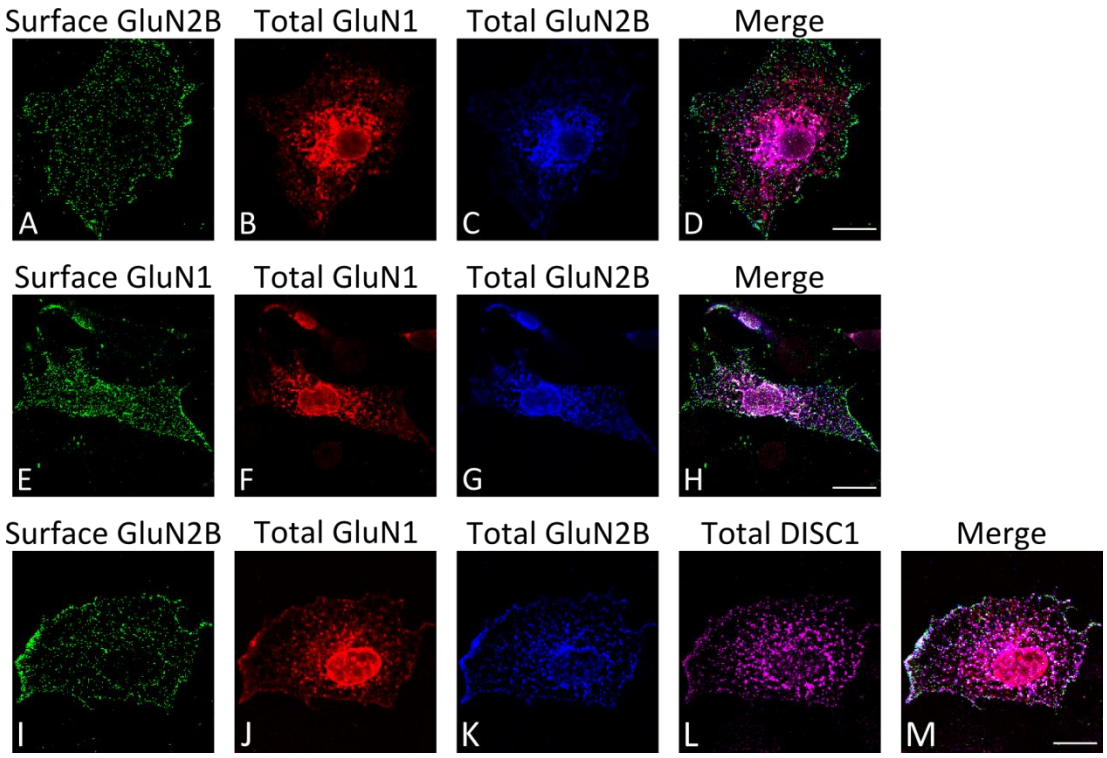

Figure 4.2 Optimisation of the surface expression assay in COS7 cells.

COS7 cells transfected with GluN1 and HA-GluN2B were stained live at 4 °C with anti-HA (A, I) or anti-GluN1 (E) to label surface NMDA receptors, then fixed, permeabilized and stained again for GluN1 (B, F, J) and GluN2B (C, G, K). When cells were also transfected with FLAG-DISC1, FLAG antibody was added post-permeabilization (L). n = 3, Scale 20 µm.

4.2.3 Confirmation of surface expression

In order to confirm the specificity of the staining of the surface-expressed NMDA receptors, COS7 cells were co-stained with VLA-2α. VLA-2α is a transmembrane receptor for collagen and other associated proteins, and is often used to indicate surface labelling (Harburger and Calderwood, 2009). COS7 cells were transfected with GluN1 and HA-GluN2B subunits and labelled live at 4 °C with an anti-HA antibody to label the surface expressed receptors. The cells were then fixed and stained for VLA-2α, GLuN1 and HA-GluN2B. Figure 4.3 A-F shows a cell with staining for endogenous VLA-2α staining (Figure 4.3 B). The surface HA-GluN2B (Figure 4.3 B) clearly co-localises with the surface marker VLA-2a (Figure 4.3 A-C).

These data confirm that the surface staining protocol for labelling GluN2B subunits gives a specific surface signal and can therefore be used to quantify the amount of signal at the surface of the cell. Furthermore total staining for GluN1, GluN2B and another protein of interest (e.g. DISC1) can also be stained for and quantified to ensure similar quantities of protein are being expressed in the cell of interest.

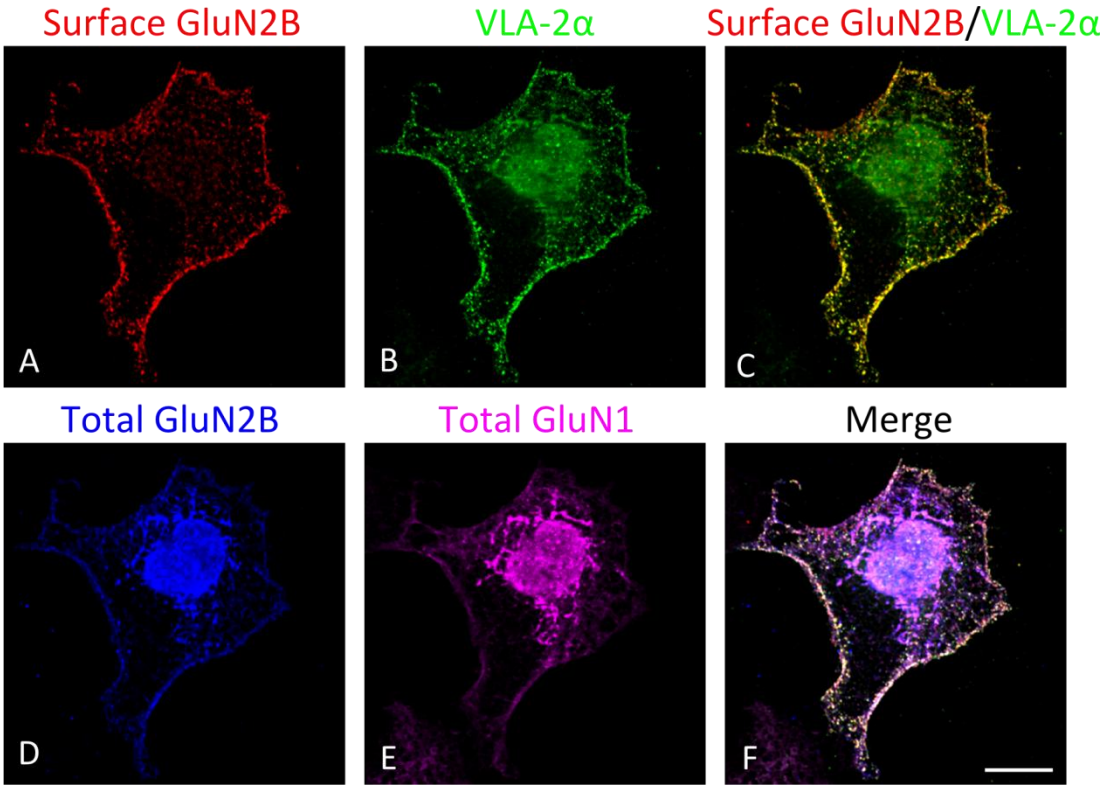

Figure 4.3. Co-localisation of surface NMDA receptors with the membrane marker VLA-2α.

COS7 cells transfected with GluN1 and HA-GluN2B were stained for surface NMDA receptors (A) and the surface membrane marker VLA-2α (B) which show strong co-localisation (C). Total HA-GluN2B (D) and GluN1 (E) show cytoplasmic and perinuclear staining patterns (F), indicating normal expression. n = 1 10 images taken Scale bar, 20 µm.

4.2.4 Surface quantification

In order to quantify the surface-expressed NMDA receptors I worked with Paul Perry (HGU, IGMM) to develop a script in I-vision. First the four colour images (Surface GluNB, Total GluN2B, GluN1 and DISC1) are manually converted to three colour images by removing the total GluN2B channel. This is due to I-vision only being able to analyse images of three colours and not four colours.

An image is opened in I-vision and is split into the three individual colour channels. A cell of interest is then selected using the surface HA-GluN2B channel by drawing a rough mask around it (Figure 4.4 A). The script crops the image to the mask and assigns a new mask around the edge of the cell, shown in green in Figure 4.4 B. This can be manually erased and drawn back in by hand if the mask is not perfect (Figure 4.4 C). The script then measures the mean pixel intensity under the green mask, thus generating a value for surface expressed NMDA receptors.

Next the script imposes the green mask over the total GluN1 image and assigns a red line around the edge of the green mask to create an outline of the cell (Figure 4.4 D). It then generates a mask around the nucleus, shown in red in Figure 4.4 D, again this can be erased and drawn back in if it is not perfect. The script then measures the mean pixel intensity between the red line and the red mask thereby generating a value for non-nuclear GluN1 expression, which can be used to determine that GluN1 levels are unaffected by the presence of DISC1. The programme then assigns the surface outline mask and the nuclear mask to the DISC1 channel (Figure 4.4 E) and measures the mean pixel intensity between the red line and the red mask thereby generating a value for non-nuclear DISC1 expression. The nucleus is omitted due to some of the DISC1 variants to be tested, DISC1-37W and DISC1-607F, not being expressed in the nucleus (Malavasi et al., 2013).

As I am interested in determining the effect of DISC1 on the surface expression of the NMDA receptor I only measured the surface NMDA receptor intensity from cells which had similar DISC1 expression levels. To ensure this, within the script a

threshold for DISC1 pixel intensity was set. Any cell expressing DISC1 below the threshold was discounted. The threshold was set by taking the average DISC1 pixel intensity from three randomly selected cells which were expressing GluN1, HA-GluN2B and had moderate DISC1 expression (i.e. no aggregation of DISC1 and moderate florescent signal) from three separate experiments. Therefore if a cell had a low DISC1 expression level the surface expression data generated from it would not be included in the analysis.

To allow direct comparison all the images within an experiment were taken within a single session and all the images were captured using the same confocal settings (laser power, gain, offset and pinhole size).

As the pixel intensity is very important in this protocol, so it is paramount there are no saturating pixels in any of the cells used for analysis as this would affect the final quantification. Before any images were taken the cells were examined, and the microscope settings were set using a random cell from one of the cover slips used in that data set. Then using the saturation indicator in NIS elements confocal imaging software, I was able to determine if any of the pixels in subsequent images had saturating pixels and if so they were discounted.

This analysis script will be used to determine if there is any effect of DISC1 or DISC1 sequence variants, NDE1 or NDE1 phosphosite mutants, or TRAK1 or a TRAK1 sequence variant on surface-expressed NMDA receptors. The script is able to quantify both the surface-expressed NMDA receptors and total DISC1 expression thus controlling for varying DISC1 expression in individual cells.

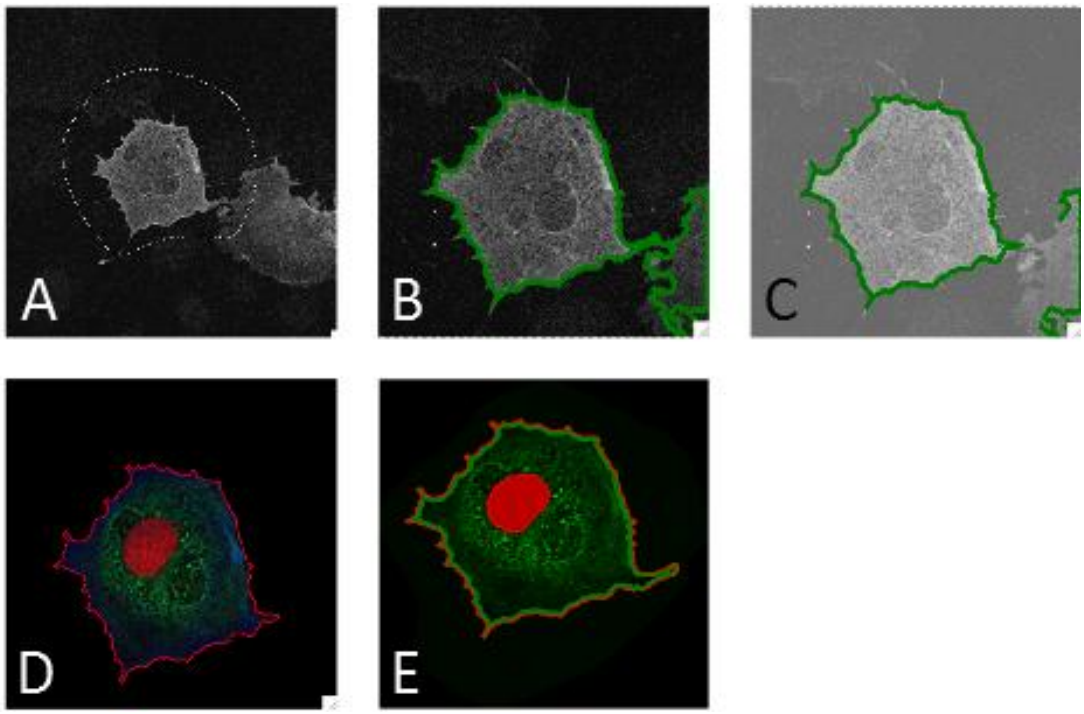

Figure 4.4. Screen shots of the surface quantification method.

A cell is selected for analysis by drawing a rough mask (dotted line) around the cell (A). A surface mask is generated by the programme and assigned around the edge of the cell of interest in green (B). This mask can be edited manually if it is not perfect (C) then the programme measures the mean pixel intensity from underneath the mask. A red line is drawn around the green mask and superimposed onto the GluN1 channel and a nuclear mask is generated in red (D). The red surface mask and the red nuclear mask are superimposed onto the DISC1 channel and the mean pixel intensity within these two masks is taken thus quantifying the levels of DISC1 (E).

4.3 Discussion

The expression and localisation of proteins is a precise and highly regulated process as mistargeting, over- or under-expression can lead to disruption of signalling cascades, intracellular trafficking, cell to cell communication and a multitude of other processes. In this chapter I set out to optimise an assay for the specific detection of surface-expressed NMDA receptors, as well as total expression levels of GluN subunits, and another protein of interest (e.g. DISC1), and to generate a script which allows for the quantification of the signal of the surface-expressed NMDA receptors and the total DISC1 expression.

COS 7 cells were chosen as a cell line to use for several reasons; they have a large size in comparison to other cell lines and therefore the subcellular structures can be better identified and analysed. Unlike other cells, COS 7 cells do not grow in a monolayer sheet but as individual cells this allows the surface of the cells to be studied as the cells will not be in close proximity to each other and the surface of the cell more accessible for antibody attachment. In cells which grow as a monolayer sheet the surface of each cell touches each other therefore knowing which surface belongs to which cell is difficult and is surface labelling is performed, there may be an accumulation of signal from two cells so measuring the surface expression via microscopy would not be possible. Finally the transfection efficiency of COS 7 cells is very high (≈ 95 %) as I will be expressing 3 or 4 constructs this gave a large number of cells which express all constructs.

The surface expression assay I described in this chapter generates a clear signal for the surface-expressed NMDA receptors. The staining protocol for GluN2B subunits at the cell surface generates a clear punctate staining pattern which is similar to that which has been published in the literature (Horak et al., 2008, Jeyifous et al., 2009). The specificity of this signal was confirmed by co-staining with a surface marker (VLA-2α). Furthermore the assay allows for the visualisation of total expressed GluN1, GluN2B and another protein (in this chapter DISC1). This ensures

only cells expressing similar amounts of exogenous protein are used for analysis as variations in surface expressed NMDA receptors could be due to varying amounts of intracellular protein. This assay gives rise to clear images of each cell and therefore allows for the analysis of each cell via a script using the imaging software I-Vision.

COS 7 cells do not endogenously express NMDA receptors but when over expressed they do form functional channels (Kaniakova et al., 2012) so NMDA receptor antagonists are used when transfecting the cells to inhibit NMDA receptor mediated excitotoxicity. DISC1, TRAK1 and NDE1 are all endogenously expressed in COS 7 cells where they may have some function within the cell. These constructs were overexpressed so to evaluate their effect on the NDMA surface expression and trafficking and evaluate more specifically their influence on NMDA receptor trafficking. There could be additional effects of the endogenous protein e.g. endogenous NDE1 expression may influence the NDE1 131A as there may be an accumulation of the effect of the over-expressed NDE1 131A and the endogenous NDE1. To determine if this happens the knockout of NDE1 and subsequent over-expression of NDE1 131A could be carried out. However as a decrease in the surface expression on NMDA receptors was observed for both NDE1 131E and DISC1 607F expressing cells there is likely to be minimal accumulation effect from endogenous protein

The translation of findings in COS7 cells into effects observed in neurons is not always the same. There is a greater degree of complexity in neurons with a larger number of interacting proteins and cell to cell interactions governing trafficking and expression of proteins. As NMDA receptors are not endogenously expressed in COS 7 cells and could be trafficked to the surface via a different mechanism than they are in neurons and may not be as constitutively active as in neurons. Conclusions made based on data generated from COS 7 cells is not always going to be the same as conclusions based on data generated using neuronal cells. However little is known about the interaction of DISC1 and the NMDA receptor or even NDE1 or TRAK1 therefore a simple cell system which can generate well controlled data is the

ideal starting point for the investigation of the effect of an interaction. Once data has been obtained from the simple system, a more complex or physiologically relevant system can be employed to further evaluate any relationship between the two interacting proteins.

Despite these caveats COS 7 cells will be suitable for the investigation of DISC1 , NDE1 and TRAK1 on NMDA receptor trafficking as they can be easily analysed as individual cells, can exogenously express numerous proteins and with further investigation of any observed effect to be confirmed in a more complex system e.g. neurons.

As I was investigating overexpressed protein the majority of the proteins I transfected in contained an epitope tag this increased the specificity of the antibody for the proteins and allowed for the easier detection of multiple proteins. Although not exactly representative of the physiological protein the FLAG-, HA-, and V5- tags are small additions to the proteins (6, 9 and 14 amino acids long respectively) and are unlikely to significantly impact on the protein function. F. Ogawa et al showed endogenous DISC1 expression and FLAG- tagged DISC1 overexpression to be very similar (Ogawa et al., 2013). Larger fluorescent tags such as GFP were not used due to their size and the causation of aggregation of proteins which made it difficult to assess the impact of DISC1 on NMDA receptor localisation and surface expression. Further to this there has been a lot of controversy regarding DISC1 antibodies (discussed in 1.1.3) and their specificity so the use of tagged constructs negated the use of any of these antibodies.

Any effect upon surface expressed NMDA receptors may be quite subtle and therefore it is important to generate a large enough data set to be able to measure these effects. Therefore three independent experiments will be carried out and 20 images for each condition (e.g. EV, DISC1) will be taken from each experiment generating 60 images per condition. The images will be taken at the same time using the same confocal settings and analysed at the same time. This will allow for the direct comparison of all the images and statistical analysis will be carried out.

The experimental n number was important as the effect of the variants could be quite subtle so a large enough sample had to be taken. 20 images were taken in each experiment and the experiment was repeated 3 times giving a total of 60 images per condition and a large enough data set for any effect to be observes. Also all images were taken at the same time which gave more consistency to the images as if they were taken over a number of days or weeks the lasers within the microscope may have deteriorated slightly or they may have been colder which could impact on their functionality and ultimately provide two images which may have the same settings on the software but are of different pixel intensity which may lead to false negative or false positive data. A larger sample size and larger independent replicates would have generated a more robust data set however, as all the images were taken at the same time and the time taken to capture all the images increasing the number of images taken would have been unfeasible.

Overexpression of trafficking proteins may be controversial as some of the proteins studied here are not endogenous to the COS-7 cell (e.g. NMDA receptors) and therefore may not follow their normal trafficking pathway. Also overexpression of endogenous proteins may overload the endogenous pathway leading the protein to behave differently and may utilise alternative pathways. On the other hand as we know that DISC1 and the NMDA receptor are likely to bind to each other it is reasonable to think the NMDA receptors use DISC1 in some capacity. As this was a novel finding there is little known about the interaction they have or what affect DSIC1 may have on the NMDA receptor so this was a first look at any potential affect that DISC1 may have or any of the variants of DISC1 may have so a simple system was used in order to determine if any interaction or effect could be seen. This could then be replicated looking at endogenous proteins to determine if the same effect is observed. As this is a novel discovery little is known about any potential impact sequence variants might have on surface expression of NMDA receptors so a “simple” system was used here.

The surface expression assay described in this chapter is a viable way to determine the effect, if any, of DISC1 and DISC1 variants, or other proteins of interest, upon NMDA receptor surface expression. The assay generates images with specific signals for surface and total expression of proteins and the script created in I-Vision allows for the quantification of these signals. This assay will therefore be used to test whether DISC1, DISC1 variants or any of DISC1 pathway partners influence the surface expression of NMDA receptors.

5 Effects of DISC1 and its pathway partners on NMDA receptor surface expression

5.1 Aims

To determine if 1) DISC1, or DISC1 carrying the variants 607F, 704C or 37W, 2) NDE1, or NDE1 phosphosite mutants, or 3) TRAK1 or a variant, putatively causal disease form, TRAK1-678R, have any effect on the surface expression of NMDA receptors in COS7 cells.

5.2 Introduction

Once released from the ER, NMDA receptors are transported to the surface of the cell at which point they are either inserted directly into the plasma membrane or maintained within an intracellular pool. Holding receptors within this pool allows for, upon activation of surface receptors, their rapid insertion into the plasma membrane (Perez-Otano and Ehlers, 2005). The exact mechanism of transport of the NMDA receptor from the ER to the surface membrane is still unknown, despite being extensively studied.

The current understanding of NMDA receptor trafficking was discussed in chapter 1. In brief NMDA receptors are trafficked via a complex of Sec8, SAP102 and mPins which attach to kinesin motors to bring the complex to the surface of the cell, however studies have shown that this is not the only mechanism of transport to the cell surface (Sans et al., 2003, Chung et al., 2004, Prybylowski et al., 2005). DISC1 could be involved in NMDA receptor trafficking due to its 1) binding directly to the GluN1 subunit of the NMDA receptor (S. Mackie unpublished), 2) direct involvement in intracellular trafficking (Shinoda et al., 2007, Taya et al., 2007, Atkin et al., 2010, Flores Iii et al., 2011, Ogawa et al., 2013) or 3) robust association with the trafficking molecules NDE1, NDEL1, LIS1, kinesin1, dynein and TRAK1 (Brandon et al., 2004, Burdick et al., 2008, Bradshaw et al., 2009, Shinoda et al., 2007, Taya et al., 2007, Kamiya et al., 2005, Ogawa et al., 2013). I therefore set out to determine whether DISC1 and some of its partners may influence NMDA receptor surface expression in COS7 cells.

To investigate this I used the trafficking assay described in chapter 4 to specifically label surface-expressed NMDA receptors, and used the computer programme

described in chapter 4 to quantify the intensity of the surface labelling, thereby allowing me to directly compare the effect of DISC1, NDE1 and TRAK1 and their respective variants and mutants, on the surface expression of NMDA receptors in COS7 cells.

5.3 Results

5.3.1 DISC1 overexpression does not influence surface NMDA receptor expression in COS7 cells

DISC1 is known to associate with the NMDA receptor as it binds directly to the GluN1 subunit (S. Mackie, unpublished) and is known to be involved in trafficking through binding to both dynein and kinesin, binding to the NDE1/LIS1/NDEL1 complex as well as an association with the trafficking molecule TRAK1 (Higginbotham and Gleeson, 2007, Shinoda et al., 2007, Ogawa et al., 2013, Taya et al., 2007, Brandon et al., 2004). To determine the effect of overexpression of DISC1 on surface expression of NMDA receptors I used the surface expression assay and the surface quantification script described earlier in chapter 3. I found that empty vector (EV)-transfected cells produce robust surface NMDA receptor expression (Figure 5.1 A) and strong total GluN2B (Figure 5.1 B) and GluN1 (Figure 5.1 C) staining. Cells co-transfected with DISC1 also gave robust surface NMDA receptor expression (Figure 5.1 E), with strong GluN1, GluN2B and DISC1 expression (Figure 5.1 F-I). Quantification of surface NMDA receptor expression revealed no significant difference in the cells expressing DISC1 compared to the EV control (as shown in Figure 5.1 J), indicating that DISC1 does not affect the amount of surface expression of the NMDA receptor in this system. Additionally the images show clear co-localisation between DISC1 and total GluN1 and GluN2B both at the surface of the cell and within the cytoplasm. This is consistent with co-IP and peptide array studies (S. Mackie and Murdoch unpublished) showing DISC1 and GluN1 interaction.

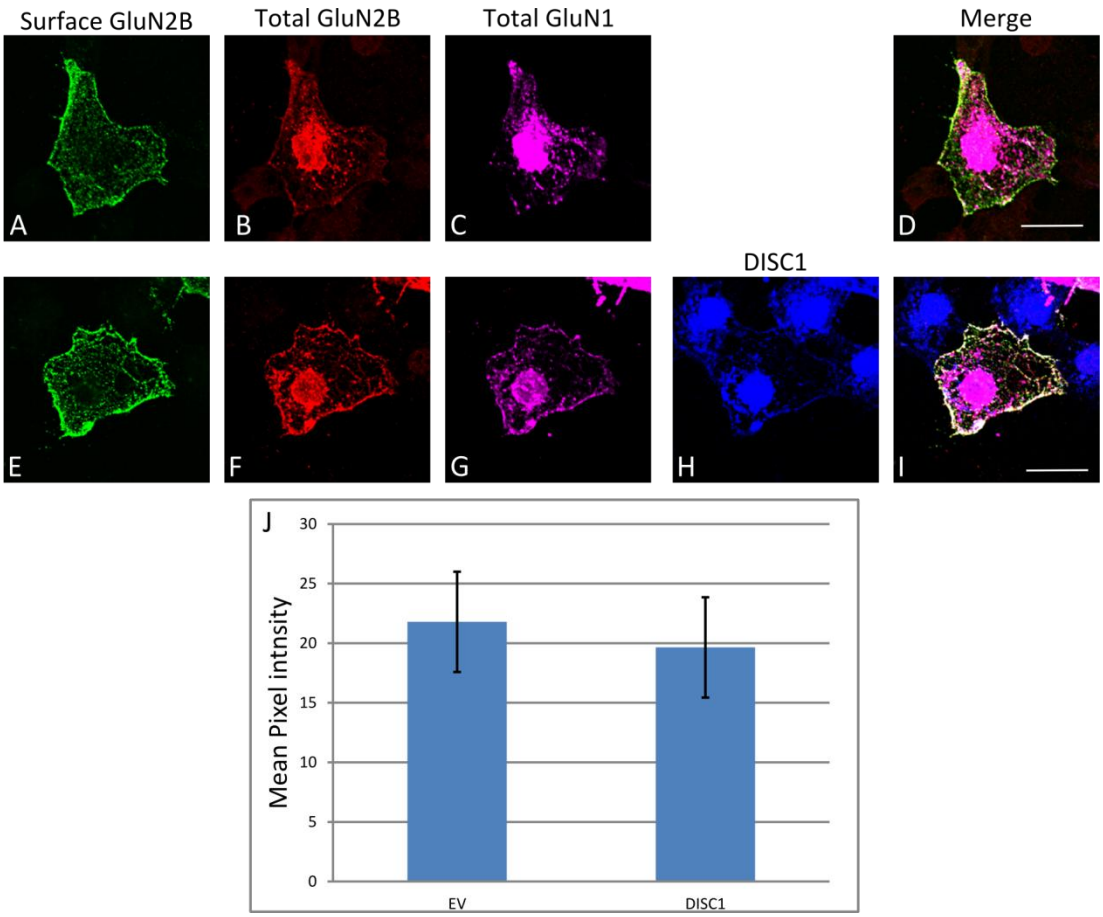

Figure 5.1 DISC1 overexpression has no effect on the quantity of surface expressed NMDA receptors.

Co-transfection with GluN1, GluN2B and either EV or DISC1 resulted in a similar mean pixel intensity after surface quantification. EV-transfected cells gave robust surface expression (A) and robust total GluN2B (B) and GluN1 expression (C), shown merged (D). Cells expressing GluN1, HA-GluN2B and FLAG-DISC1 also gave robust surface staining (E) and robust total Glun2B (F), GluN1 (G) and DISC1 (H) staining patterns, shown merged (I). J shows quantification of surface expression, n=3 independent experiments, 20 images per experiment, total=60 cells. No significant difference was observed between cells transfected with EV or DISC1. Error bars represent SEM, Scale 20 µm. C and G were pseudocoloured magenta from gray using ImageJ.

5.3.2 DISC1 variant 607F causes a reduction in surface expression of NMDA receptors

As DISC1 had no effect on surface expression of NMDA receptors, I next investigated whether any of the DISC1 variants have an effect on surface expression. COS7 cells were transfected and stained as described above. Cells expressing DISC1 again resulted in robust surface expression of NMDA receptors (Figure 5.2 A) with strong total staining for total GluN1 (Figure 5.2 C), GluN2B (Figure 5.2 B) and DISC1 (Figure 5.2 D). However in cells expressing DISC1 carrying the variant L607F (DISC1-607F) the surface expression of NMDA receptors is greatly reduced as determined using the t-test (F, $p=0.02$) with the total staining for the proteins remaining similar to that observed with DISC1 (Figure 5.2 G,H,I). The variants 704C and 37W did not generate any significant differences in the surface expression of NMDA receptors (Figure 5.2 K-T) when compared to DISC1 expressing cells. Furthermore quantification of DISC1 signal is similar across all the constructs (Figure 5.2 V) indicating a true effect of DISC1-607F rather than an effect of unequal expression of the various DISC1 species.

Notably, the variant forms of DISC1 used in these experiments co-localise with total GluN1 and GluN2B to the same extent as wild-type DISC1.

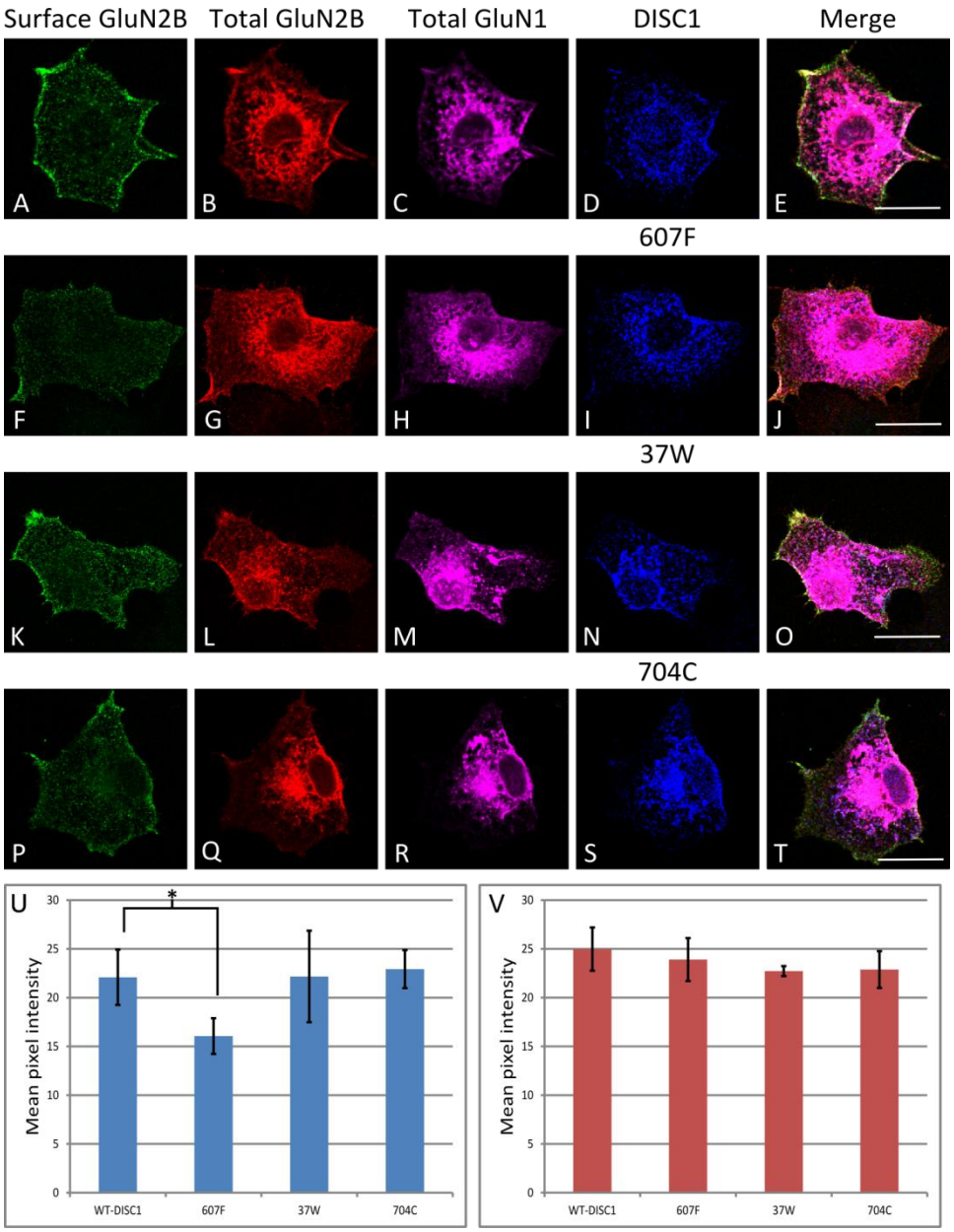

Figure 5.2. DISC1-607F decreases the surface expression of NMDA receptors compared to wild-type DISC1.

COS7 cells expressing GluN1, HA-GluN2B and either DISC1, DISC1-607F, DISC1-704C or DISC1-37W were surface stained for NMDA receptors and then stained for total expression of transfected protein. DISC1 expressing cells (A-E) show robust surface staining (A) with strong total expression of GluN2B (B), GluN1 (C) and DISC1 (D and merge E). DISC1-607F expressing cells (F-J) exhibit a decrease in surface expressed NMDA receptors (F) with similar amounts of total protein still being expressed (G-I merge J). Quantification of the surface-expressed NMDA receptors showed a statistically significant decrease in the presence of DISC1-607F in comparison to DISC1 ($p=0.02$, U). Cells expressing DISC1-704C (K-O) or DISC1-37W (P-T) showed no difference in surface-expressed NMDA receptors (U). Finally, quantification of DISC1 and variant DISC1 showed no significant difference (V). Error bars represent SEM, Scale 20 µm, n=3 independent experiments, 20 images per experiment, total=60 cells. Statistical analysis of DISC1 vs DISC1-607F was carried out using a paired two-tailed t-test. C, H, M and R were pseudocoloured magenta using ImageJ

5.3.3 DISC1 and L607F-DISC1 do not affect whole cell NMDA-induced current in neurons

I next determined if DISC1 or DISC1-607F transfected neurons exhibit any differences in whole cell NMDA-induced currents. Based on the decreased surface NMDA receptor expression induced by DISC1-607F in COS7 cells, I wanted to determine if there is a functional effect upon NMDA receptors in neurons. Cortical neurons were plated on glass coverslips and transfected at DIV 8-9 with GFP (to visualise transfected neurons) and either DISC1, DISC1-607F or globin (as a control). Whole cell NMDA receptor recordings were made between DIV 10-11. 13 recordings were made from cultures from 5 different animals (as described in (McKay et al., 2012)). The recordings were normalised to the cell capacitance and the current density was calculated. There was no significant difference in the capacitance of the transfected cells which indicated that the cells which recordings were obtained from were of similar size and any differences observed are due to differences in NMDA receptor density.

Globin transfected cells had an average current density of 47.15 pA/pF (Figure 5.3 A). DISC1 transfected cells gave a similar current density of 40.73 pA/pF (Figure 5.1Figure 5.3 A), there was no significant difference between globin and DISC1 transfected cells as determined by t-test. DISC1-607F transfected cells had a similar current density of 43.67 pA/PF (Figure 5.3 A) which was not significantly different from DISC1.

Although there is an indication that DISC1 and DISC1-607F do not affect the global population of surface expressed NMDA receptors in cortical neuron, more detailed investigations will be required for any firm conclusions to be drawn, which is discussed in 5.4.

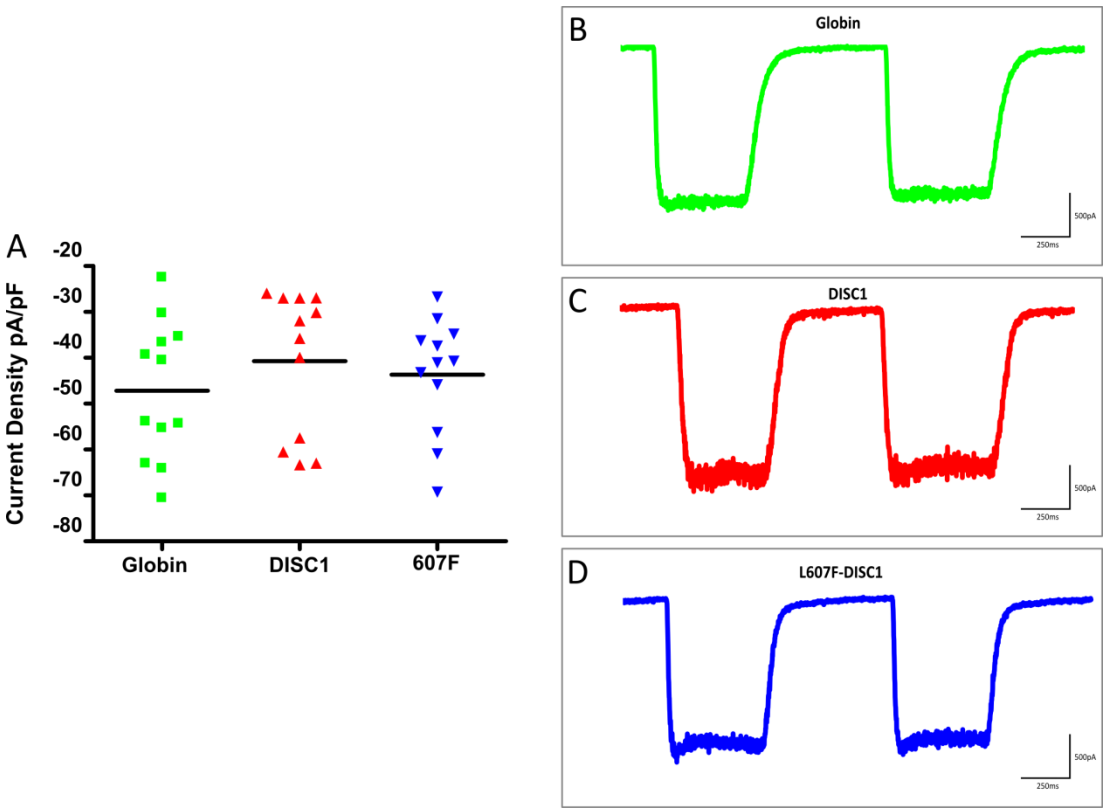

Figure 5.3 DISC1 and DISC1-607F have no effect on current density in pyramidal neurons

Cortical neurons were transfected between DIV 8-9 and whole cell NMDA recordings were taken DIV 10-11. Neurons were maintained in Mg2+ free recording solution and clamped at -70 mV. A whole cell patch was formed and a baseline recording was taken. Cells were then washed in NMDA to open NMDA receptors causing a drop in current density. The NMDA was washed from the cells by applying the Mg2+ free recording solution. This was repeated a further 3 times. An average reading of the baseline when in Mg2+recording solution was taken and an average reading of the baseline when NMDA is applied was taken and these values were subtracted from each other. These were then divided by the cell capacitance to generate the current density.

Graph A shows the comparison of globin, DISC1 and DISC1-607F whole-cell NMDA currents evoked by 300 µm NMDA. B, C and D are example traces used to generate data in A , n=5 independent experiments, 12 recordings in total

5.3.4 NDE1 and NDE1 phosphomutants modulate the surface expression of NMDA receptors

I next investigated the effect of NDE1 on surface expression of NMDA receptors. COS7 cell transfection with GluN1, HA-GluN2B and EV leads to robust surface expression of the NMDA receptor (Figure 5.4 A) as well as strong total expression of GluN1 and GluN2B, as expected (Figure 5.4 B-C). Cells co-expressing NDE1 generally exhibit about the same, or slightly increased, surface expression of NMDA receptors as EV-transfected cells (Figure 5.4 E). NDE1 co-expression also does not affect total GluN1 and GluN2B expression (Figure 5.4 F-G). Co-transfection with NDE1-131A, leads to slightly increased surface NMDA receptor expression (Figure 5.4 J), again without affecting total GluN1 or GluN2B total expression (Figure 5.4 K,L). The phospho-mimic mutant NDE-131E decreases surface NMDA receptor expression (Figure 5.4 O) without affecting total expression levels of GluN1 (Figure 5.4 Q) and GluN2B (Figure 5.4 P). Importantly, expression of all three forms of NDE1 was similar (Figure 5.4 U). Analysis of the data using one-way ANOVA found an overall effect ($p=0.047$) of NDE1 or phospho-NDE1 on the surface expression of NMDA receptors. Post-hoc pairwise Bonferroni testing revealed was due to a significant difference between NDE1-131A and NDE1-131E upon NMDA receptor surface expression ($p<0.05$). Individual pairwise Bonferroni testing also revealed a significant difference between NDE1 and NDE1-T131E ($p<0.05$), but this did not survive correction for multiple comparisons.

There was also co-localisation between NDE1 and total GluN1 and total GluN2B staining, which indicates that the NMDA receptor and NDE1 might be able to associate within COS7 cells. There is some evidence potentially linking NDE1 and NMDA receptors as NDE1 has been shown to localise at excitatory synapses (Bradshaw et al., 2008).

Taken together this indicates that NDE1 can modulate the amount of surface expressed NMDA receptors in COS7 cells, with phosphorylation at T131 potentially being an important regulatory event.

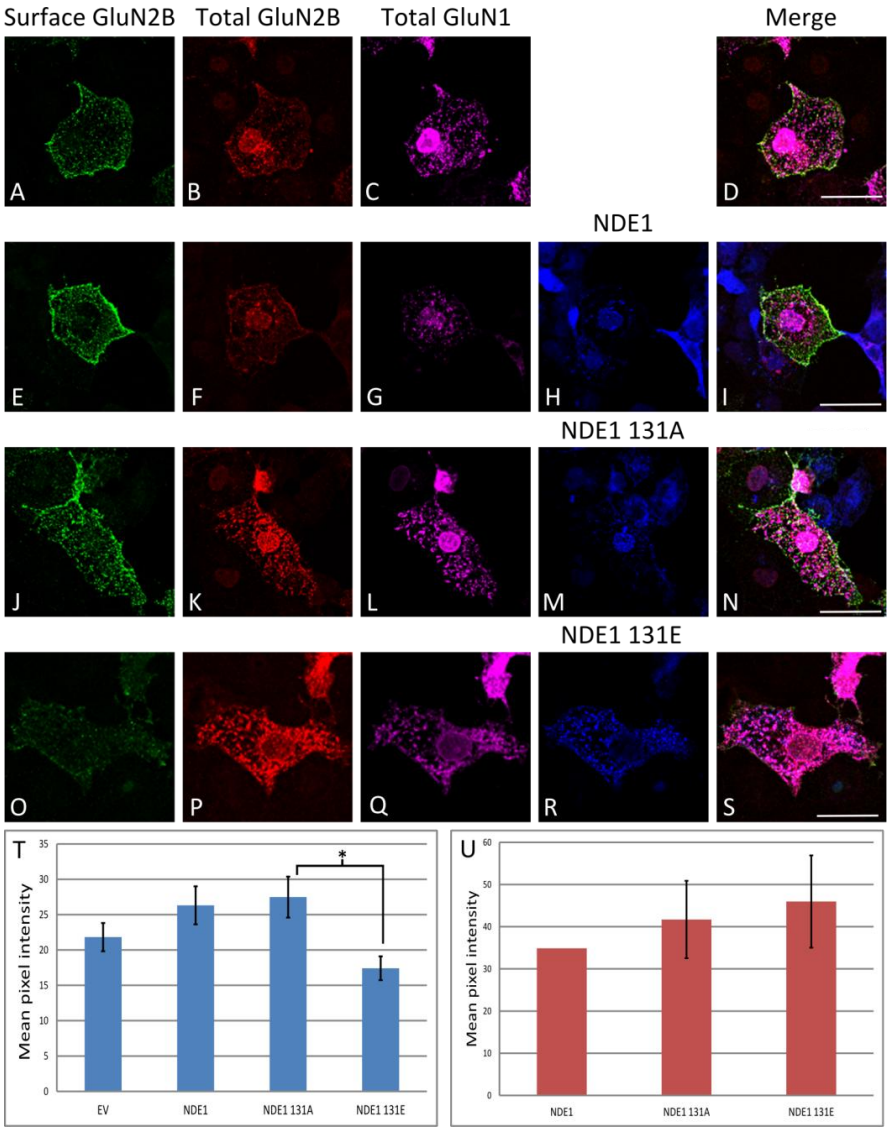

Figure 5.4. Effect of NDE1 upon NMDA receptor surface expression.

COS7 cells co-transfected with GluN1, GluN2B and EV generated robust surface staining (A) with strong total staining of GluN2B (B) and GluN1 (C), merged in (D). Cells transfected with GluN1, GluN2B and NDE1 showed slightly stronger surface expression of NMDA receptors (E) while giving similar total staining of GluN2B (F) and GluN1 (G) as EV-transfected cells, and robust NDE1 staining (H merged in I). Similarly, cells exogenously expressing GluN1, GluN2B and NDE1-131A exhibited slightly increased NMDA receptor surface expression (J) while generating similar GluN2B (K), GluN1 (L) and NDE1-131A (M merged in N) signals, as EV or NDE1-transfected cells. Finally cells exogenously expressing GluN1, GluN2B and NDE1-131E gave decreased NMDA surface expression (O) whilst still generating similar GluN2B (P), GluN1 (Q) and NDE1-131A signal (R merged in S). Quantification of the surface-expressed NMDA receptors showed a statistically significant decrease in the presence of NDE1-131E in comparison to NDE1-131A ($p<0.05$, T). Finally, quantification of NDE1 and phosphomutant NDE1 expression showed no significant difference (U). Error bars represent SEM, n=3 independent experiments, 20 images per experiment, total=60 cells Scale 20 µm. Statistical analysis of NDE1 and phosphomutant NDE1 was carried out using one-way ANOVA with post-hoc pairwise Bonferroni testing. C, G, L and Q were pseudocoloured magenta from gray using ImageJ

5.3.5 TRAK1 decreases NMDA receptor surface expression

As reported in Chapter 4 the trafficking molecule TRAK1 co-localises robustly with GluN2B so I was interested in using TRAK1, and TRAK1 carrying a putative mutation H678R, identified from a patient with schizophrenia (Xu et al., 2011), in the trafficking assay. COS7 cells co-expressing GluN1, HA-GluN2B and EV gave robust surface staining (Figure 5.5 A) and strong total expression of GluN1 and GluN2B (Figure 5.5 B-D). Conversely, COS7 cells co-transfected with GluN1, HA-GluN2B and TRAK1 generated very weak NMDA receptor surface staining (Figure 5.5 E-I). However as expected, total staining of GluN1 and GluN2B in cells exogenously expressing TRAK1 was different to that in EV-transfected cells as the receptor subunits appeared to be redistributed to TRAK1-positive aggregations (Figure 5.5 H), most likely at the mitochondria. TRAK1-678R also decreased the number of surface expressed NMDA receptors and caused GluN1 and GluN2B clustering (Figure 5.5 J-N). One way ANOVA revealed a significant effect ($p=0.009$) when comparing surface NMDA expression in EV or TRAK1 or TRAK1-678R, which post hoc Bonferroni pairwise testing revealed was due to a significant decrease in NMDA receptor surface expression in the presence of TRAK1, with no difference between TRAK1 and TRAK1-678R (O). Thus the mutation does not have a significant effect on TRAK1-induced reduction of NMDA surface expression in COS7 cells. Finally the expression of TRAK1 and TRAK1-678R was approximately the same, indicating again that the lack of effect of the mutations is not due to different expression levels.

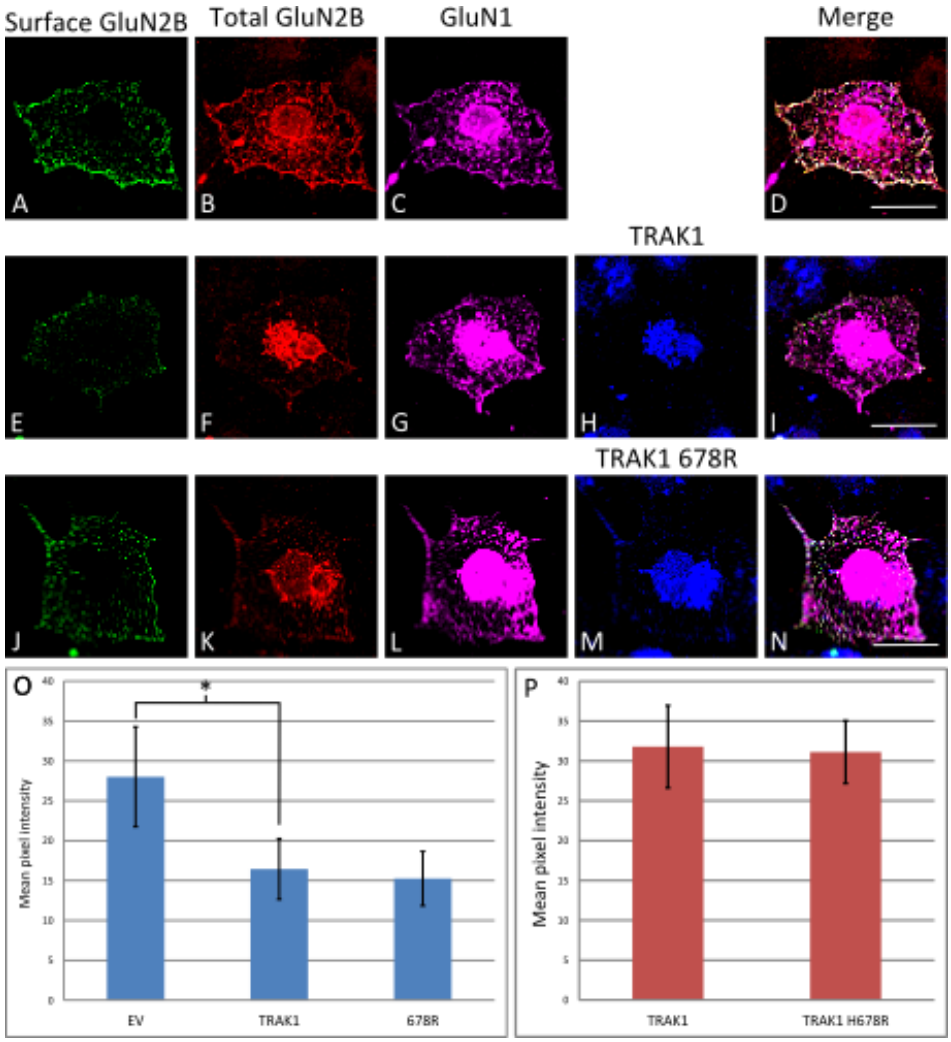

Figure 5.5. Co-expression of TRAK1 or TRAK1-678R causes a significant decrease in surface expressed NMDA receptors.

COS7 cells expressing GLuN1 GluN2B and EV generated robust surface expression of NMDA receptors (A) and strong total gluN2B (B) and GluN1 (C), merged in (D) expression. Cells exogenously expressing GluN1, GluN2B and TRAK1 show much reduced NMDA receptor surface expression (E) with total staining for both total GLuN2B (F) and GluN1 (G) appearing with a similar staining pattern to mitochondria as described in chapter 3, along with TRAK1 (H), merged in (I). Similarly in cells co-transfected with GluN1, GluN2B and TRAK1-678R reduced surface NMDA expression (J) was observed with total staining of GluN2B (K), GluN1 (L) and TRAK1-678R (M), merged in (N) having a similar appearance to mitochondrial staining as described in chapter 3. Scale 20 µm, n=3 independent experiments, 20 images per experiment, total=60 cells.

Quantification of surface expression (O) using one-way ANOVA shows a statistically significant decrease in NMDA receptor surface expression in cells expressing TRAK1 ($P=0.03$). Post hoc testing revealed a significant decrease in surface NMDA receptor expression in the presence of TRAK1 ($p<0.05$), but no statistically significant difference between TRAK1 and TRAK1-678R expressing cells. Quantification of TRAK1 expression (P) shows no significant difference in expression between wild-type and mutant. Error bars represent SEM. * $p<0.05$, 20 cells counted per experiment, 60 in total. C, G and L were pseudocoloured magenta from gray

5.4 Discussion

The precise mechanism of how NMDA receptors are trafficked from the ER to the surface is not fully understood. The C-terminal PDZ domain binds and interacts with SAP102, mPins and Sec8 and travels along microtubules to the surface membrane (Lau and Zukin, 2007, Sans et al., 2003, Sans et al., 2005). However removal of the PDZ domain of the GluN2B subunit disrupts SAP102 binding but does not inhibit surface expression of GluN2B containing NMDA receptors at the cell surface, which indicates another method of intracellular trafficking (Sans et al., 2003).

The results in this chapter show wild-type DISC1 has no effect on the surface expression of the NMDA receptor, at least in COS7 cells. However the DISC1 variant 607F was found to cause a significant reduction in surface expressed NMDA receptors when compared to wild type DISC1. Furthermore neither the 704C nor 37W variants had any effect on surface expression. As DISC1 is known to bind directly to GluN1 (S. Mackie unpublished) and is vital in many different trafficking processes (e.g. transport of GRB2, mitochondria and synaptic vesicles and also part of the LIS1/NDE1/NDEL1 trafficking complex, (Atkin et al., 2010, Taya et al., 2007, Shinoda et al., 2007, Ogawa et al., 2013, Flores Iii et al.), it was surprising to find its overexpression doesn't affect the surface expression of NMDA receptors. However because DISC1-607F causes a decrease in surface expression, it is reasonable to think DISC1 does play a role in some capacity, which is disrupted by the 607F variant form. It has been reported that DISC1-607F fails to rescue mitochondrial trafficking deficits induced by DISC1 knock down, whereas both wild type DISC1 and DISC1-704C can (Atkin et al., 2010), indicating some loss of function of the 607F variant. Furthermore DISC1-607F has been associated with lower levels of PCM1 at the centrosome (required for correct axonal morphology and embryonic neurogenesis (Bradshaw and Porteous, 2012), lower levels of DISC1 in the nucleus and disruption of ATF4 mediated transcription (Malavasi et al., 2013), indicating that DISC1-607F can cause disruptions to correct DISC1 function.

As DISC1 is largely a brain expressed protein it would be interesting to carry out these experiments in neurons. There may be several mechanisms operational in neurons which are not present in non-neuronal cell lines which could influence any role DISC1 may have in NMDA receptor trafficking. The Millar group has shown that the 607F variant affects receptor movement in axons of hippocampal neurons. Time lapse imaging showed that in neurons co-transfected with GluN1 and DISC1 the 607F variant significantly decreases the velocity of movement of GluN1-containing vesicles. Although these studies were not directly measuring surface expression they further strengthen the evidence for a role of DISC1 in NMDA receptor trafficking and for the common variant L607F having an impact on this.

The other DISC1 variants, 704C and 37W, have been shown to induce disruptions to DISC1 function. 704C has also been shown to induce lower levels of PCM1 at the centrosome (Eastwood et al., 2010), a mild reduction in DISC1 binding to NDE1 and NDEL1, and altered DISC1 oligomeric status (Burdick et al., 2008). 37W has been shown to alter the mitochondrial distribution of DISC1 and induce mitochondrial morphological abnormalities (Ogawa et al., 2013). Interestingly neither caused any disruption to the surface expression of NMDA receptors. One possibility is that the 607F variant, but not 37W or 704C, disrupts DISC1 binding to the NMDA receptor. Structural analysis predicts that 607F is likely to impact on the structure of DISC1 as it lies within a leucine zipper and could disrupt the leucine-leucine packing between adjacent helices of the zipper, thereby modulating the coiled-coil stability, affecting oligomeric state, protein partner selection and the orientation of coiled-coil helices (Soares et al., 2011). The 37W and 704C variants, however, are not predicted to alter DISC1 structure, although they do impact on its protein-protein interactions (Soares et al., 2011).

Based on the results showing DISC1-607F decreases the surface expression of NMDA receptors in COS7 cells, whole cell recordings of NMDA currents were taken from cortical neurons transfected with globin, DISC1 or DISC1-607F. Based on the recordings made in Figure 5.3 DISC1 transfected cells showed no significant

difference when compared to globin transfected cells. This was to be expected based on the data shown in Figure 5.1 which indicates that DISC1 does not cause a significant difference in the surface expression of NMDA receptors. However there was also no significant difference in current density detected between DISC1 and DISC1-607F transfected cells which is not in agreement with the data presented in Figure 5.2. This could be due to the differences in model cell used as there is most likely additional complexity within the trafficking mechanism of surface expression of NMDA receptors in neurons which is not present in COS7 cells. For example 300nm NMDA was applied to stimulate the neurons, this is a saturating concentration of NMDA which stimulated both synaptic and extra-synaptic NMDA receptors. DISC1 has been shown to localise to extra-synaptic sites (Paspalas et al., 2012) and therefore could affect the trafficking of NMDA receptors at either one of these sites. This could be investigated by blocking synaptic NMDA receptors and measuring the effect of overexpressed DISC1 or DISC1-607F or DISC1 knockdown. Furthermore in the COS7 assay the levels of NMDA surface expression is being measured at homeostasis i.e. not being stimulated whereas in neurons there is a much greater activity of the receptor. For example during synaptic plasticity there are rapid changes in surface expressed NMDA receptors, DISC1 could play an important role in the transport of the NMDA receptor but this was not captured in these experiments. Knockdown of DISC1 and subsequent patch clamping of neurons would be the next logical step.

DISC1-607F has been shown to affect the trafficking of NMDA receptors in axons (Malavasi unpublished) and fails to rescue mitochondrial trafficking deficits in axons (Atkin et al., 2010). Therefore evidence suggests that DISC1 is involved in NMDA receptor trafficking, and that this mechanism may be affected by the 607F variant, so measuring the effect of this variant at a more specific point in the trafficking mechanism may be more informative than the whole cell current recordings performed here. DISC1 has been shown to interact with dynein and kinesin which may link DISC1 to receptor endocytosis as these molecular motors have been shown to play a critical role in trafficking of the NMDA receptor to and from the cell

surface (Guillaud et al., 2003, Wang et al., 2010a, Setou et al., 2000). Moreover the robust association of DISC1 with TRAK1 also suggest a potential role in receptor trafficking because TRAK1 is already known to associate with endosomes and mediate receptor trafficking (Gilbert et al., 2006, Webber et al., 2008). It would be interesting to determine if DISC1 does play a role in receptor endocytosis and if the 607F variant has an effect on this as well. Furthermore, due to the complexity of NMDA receptor signalling it could be that in cells co-expressing DISC1-607F there is no overall reduction in current density, but there could still be less receptors expressed at the surface, with the ones that are expressed compensating by being more active. Alternatively any effect on NMDA receptor trafficking by DISC1-607F could be investigated via long term potentiation (LTP) or long term depression (LTD) studies as this would measure the influence of DISC1 and DISC1-607F on NMDA receptor insertion to synapses over time: if the 607F variant is affecting the mechanism of receptor trafficking to or from the cell surface then it may become apparent in this kind of study.

NDE1 binding has been shown to determine correct dynein function (Lam et al., McKenney et al.). NDE1 phosphorylation has been shown to decrease its binding to dynein, disrupting dynein function and subsequently affecting intracellular trafficking (Hirohashi et al., 2006a). My study showed NDE1 and the phospho-null mutant NDE1-131A had no effect on surface NMDA receptor expression. However the phospho-mimetic NDE1-131E causes a significant decrease in surface expressed NMDA receptors when compared to NDE1-131A-expressing cells. This indicates NDE1 may be involved in regulating the surface expression of NMDA receptors in COS7 cells, but in a phospho-dependent manner. Phosphorylation of NDE1 at T131 has been shown to disrupt NDE1/LIS1 binding, which could cause deficits in trafficking through misregulation of dynein activity (Bradshaw et al., 2011). NDE1/LIS1 interaction promotes dynein processivity (McKenney et al., 2010) and therefore phosphorylation at T131 may block dynein function resulting in trafficking deficits. Blocking dynein function may not explain the reduction in surface expressed NMDA receptors as dynein is a retrograde transporter and takes

organelles away from the cell periphery. Blocking this function would be predicted to result in an increase in cell surface expression (as transport away from the surface has been disrupted), therefore there may be another mechanism disrupted which results in the reduction of surface expressed NMDA receptors. The LIS1/NDE1/NDEL1 complex is also essential for dynein-dependent organelle positioning, which includes the Golgi, early endosomes and lysosomes (Lam et al., 2009). Therefore disruption to this complex may affect Golgi positioning which could result in decreased trafficking of NMDA receptors to the surface, or it may be that disruption to this complex directly affects the trafficking mechanism of the NMDA receptor, resulting in reduced trafficking.

Interestingly phosphorylation of NDE1 at T131 is controlled by DISC1, therefore potentially linking DISC1 and NDE1 in the trafficking of NMDA receptors (Bradshaw et al., 2011). DISC1 upregulates phosphorylation of NDE1 at T131 (Bradshaw et al., 2011), which should result in overexpression of DISC1 causing a reduction in surface expression of NMDA receptors, which is not the case (chapter 5.3.1). This may be because the levels of phosphorylation of NDE1 are most likely not 100% in the presence of overexpressed DISC1, in contrast to overexpression of NDE1-131E which should mimic 100% phosphorylation at position 131. Also there could be an alternative mechanism which can compensate for any deficit in trafficking resulting from increased NDE1 phosphorylation after DISC1 overexpression

Finally, TRAK1 was found to significantly decrease the surface expression of NMDA receptors in COS7 cells. This is most likely due to TRAK1 sequestering the receptors at the mitochondria. This was observed as total staining patterns of GluN1 and GluN2B revealed a different staining pattern compared to that in EV-transfected cells, as they co-localised with overexpressed TRAK1. In 3.4 TRAK1 was found to alter GluN2B subcellular distribution by sequestering some of it at the mitochondria. Further to this TRAK1 had no effect on GluN1 subcellular distribution when expressed without GluN2B, but when the subunits are co-expressed TRAK1 sequesters both at the mitochondria indicating a strong association with GluN2B

and assembled NMDA receptors, which recruits GluN1 to the mitochondria. I speculate that this could lead to a decrease in the surface expressed NMDA receptors as they are being held at the mitochondria and thereby cannot be inserted into the plasma membrane. TRAK1 mutant 678R also had this effect, perhaps indicating the mutation has no effect on TRAK1s ability to sequester the NMDA receptor at the mitochondria. However, although the effect I observed upon NMDA receptor surface expression may be an artefact of TRAK1 overexpression there is clearly a robust association between TRAK1 and GLuN2B that is worthy of further investigation.

Although not investigated here it would be interesting to see what effect TRAK1 knockdown has on NMDA surface expression. As overexpressed TRAK1 is largely mitochondrial, endogenous TRAK1 has been shown to be additionally localised to early endosomes (Webber et al., 2008) and therefore could be involved in receptor recycling. Knock down of TRAK1 could increase surface expression through lack of endocytosis of inserted receptors or could cause a decrease in surface expressed receptors by blocking recycling of receptors as it does with EGF stimulated EGFR degradation (Webber et al., 2008).

Improvements could have been made to the methods although the script used was specifically designed for the analysis of these experiments as a way to determine if effects which may be subtle to the eye could be analysed. The quantification of the surface intensity of the NMDA receptor provided a numerical value to the level of staining at the surface so any observed effect of due to a particular overexpressed protein could be determined. This could have been improved by taking a Z-stack of the image and quantifying each layer of the cell to build up a 3D image of the cell and it surface expression levels. This, however the time taken to acquire all these images would have been much greater so to take all the images from each experiment on the same day would result in a lower number of replicates and ultimately less confidence in the results generated. New super-resolution microscopes could improve the image quality and allow for greater clarity of any co-

localisation and the location within the cell of that co-localisation however I have looked at the surface expression rather than the co-localisation of two proteins in the results of this chapter so acquiring super-resolution images may not add much more information to the image.

The lab already has data demonstrating that endogenous DISC1, TRAK1 and NDE1 can be co-immunoprecipitated from brain, thus it is possible that these proteins form a complex that regulates NMDA receptor surface expression. This function could be influenced by the DISC1 sequence variant 607F: for example altered binding efficiencies could lead to decreased trafficking complex formation and therefore decreased surface expression. Furthermore the complex could also be regulated by phosphorylation events on NDE1 which cause disruption of the complex.

Additionally DISC1, NDE1 and TRAK1 are all expressed in neurons (Bradshaw et al., 2008, Hayashi-Takagi et al., van Spronsen et al.), so it would be interesting to determine if NDE1 or TRAK1 or the DISC1 variants have any effect on NMDA receptor surface expression in neurons. I showed here that in COS7 cells they may be involved in the trafficking of the NMDA receptor and showed in chapter 4 co-localisation of; TRAK1, GluN1 and GluN2B; NDE1, GluN1 and GluN2B; and DISC1, GluN1 and GluN2B subunits in hippocampal neurons. Therefore it is conceivable that they form a complex in neurons and may affect NMDA receptor surface expression, however the NMDA receptor is a highly dynamic receptor which is constantly inserted and endocytosed from the membrane so additional information about how these proteins modulate surface NMDA receptor expression is needed, do they alter the trafficking from the ER? Or do they affect the endocytosis when the NMDA receptor is internalised? Or can they affect the re-insertion of the receptor into the plasma membrane? Some of this will be the focus of the next chapter.

Altogether these results show that wild type DISC1 and the variants 704C and 37W do not affect the surface expression of the NMDA receptor. The DISC1 variant 607F

significantly decreases surface expression in COS7 cells as do NDE1-131E, TRAK1 and TRAK1-678R. Thus DISC1 and its binding partners have the ability to modulate the surface expression of the NMDA receptor in COS7 cells.

6 Investigating a role for DISC1 and NDE1 in NMDA receptor endocytosis

6.1 Aims

To determine if DISC1,DISC1-L607F,NDE1, or NDE1 phosphosite mutants have any effect upon surface-labelled NMDA receptor internalisation in COS7 cells.

6.2 Introduction

6.2.1 NMDA receptor endocytosis

Endocytosis is a fundamental mechanism by which cells regulate intracellular signalling, cell to cell communication, synaptic strength and synaptic maturation (amongst many other things). In the case of the NMDA receptor, endocytosis occurs by the assembly of clathrin coated vesicles and budding of clathrin coated vesicles from the plasma membrane. This process is tightly regulated and governed by subunit-specific motifs.

The GluN2B subunit can bind directly to PSD95, via its PDZ domain, which stabilizes the NMDA receptors at the surface membrane and suppresses GluN2B mediated internalisation. Deletion of the PDZ domain in the GluN2B subunit significantly increases internalisation of NMDA receptors. GluN2B contains an internalization signal (YEKL) within the distal C-terminus (Roche et al., 2001). This signal is part of a well characterised family of tyrosine-based internalisation signals present in the membrane proteins (Bonifacino and Dell'Angelica, 1999). This motif acts as a recognition motif for the endocytic adaptor protein AP2 linking the NMDA receptor to clathrin coated vesicles (Lavezzari et al., 2003, Roche et al., 2001, Carroll and Zukin, 2002). Deletion of the YEKL signal in GluN2B inhibits GluN2B internalisation (Roche et al., 2001).

After internalisation the NMDA receptor localises with transferrin and therefore the early endosome. Once the receptor has been internalised, there is a subunit specific intracellular trafficking pathway. GluN1/GluN2B homodimers have been shown to predominantly follow a recycling pathway as the receptors localise to RAB5 before localising to RAB11 (Takahashi et al., 2012). The receptor can then be re-inserted into the plasma membrane, which all occurs within 30 minutes (Lavezzari et al., 2003, Lavezzari et al., 2004, Tang et al., 2010), and the receptor is

thought to be continuously cycled through this pathway. GluN1/GluN2A homodimers predominantly follow a degradative pathway as they initially co-localise with RAB5 before co-localising with RAB7, which is a marker for the late endosome (Lavezzari et al., 2003, Lavezzari et al., 2004, Tang et al., 2010), and therefore are thought to be predominantly degraded after internalisation.

6.2.2 DISC1, NDE1 and TRAK1 in recycling

Although not directly investigated yet, DISC1 has been reported to co-localise with RAB11 in neurons, opening up a potential role in recycling of receptors (Lepagnol-Bestel et al., 2013). Furthermore the authors of this paper also found DISC1 not to co-localise with RAB7 indicating that DISC1 may not play a role in degradation pathways. However, as we shall see later, my more in-depth study has not replicated this latter observation.

Currently NDE1 has not been shown to be directly linked to any RAB proteins, but NDE1 is known to regulate the positioning of organelles, with knockdown of NDE1 causing slight mis-positioning of endocytic compartments and depletion of dynein from membranes, both of which could affect the internalisation of cell surface proteins (Lam et al., 2009). Also, recently RAB11 (through an interacting protein RAB11-FIP3) was shown to link to dynein and mediate transport of material from peripheral sorting endosomes to the recycling endosome (Horgan et al., 2010). This potentially links NDE1 to RAB11 and recycling pathways as the NDE1/LIS1/NDEL1 complex is required for dynein function.

Taken together the literature suggests DISC1 and NDE1 could play a role in receptor endocytosis and, as I have previously described, they both associate with the NMDA receptor and can alter the surface expression of NMDA receptors in COS7 cells. In this chapter I use an internalisation assay to determine the effect of DISC1 or NDE1 on the endocytosis of NMDA receptors by studying co-localisation of the NMDA receptors and RAB5 (endocytic vesicle/early endosome) or RAB7 (late endosome) markers.

6.3 Results

6.3.1 Optimisation of the internalisation assay

Fluorescent mCherry-tagged RAB5 and RAB7 were used to determine if DISC1 or NDE1 play a role in NMDA receptor endocytosis or degradation. In the basic assay COS7 cells are transfected with plasmid constructs expressing GluN1, HA-GluN2B plus mCherry-RAB5 or mCherry-RAB7. The cells are stained live with an anti-HA antibody at 4 °C to label surface-expressed NMDA receptors, fixed, and stained for total GluN1. Under these conditions there is little co-localisation between surface HA-labelled NMDA receptors and the intracellular fluorescently tagged RAB proteins. To examine endocytosis, after surface HA-labelling the cells are incubated at 37 °C which allows HA-tagged receptor internalisation. The incubation time is critical because a 15 minute incubation leads to surface-labelled receptor/RAB5 co-localisation, and a 30 minute incubation allows the endocytic pathway to proceed further leading to surface-labelled receptor/RAB7 co-localisation (Scott et al., 2004, Lavezzari et al., 2004, Tang et al., 2010). These published studies were carried out using COS7 cells and are therefore applicable to this assay. For this assay I will use a 15 minute incubation at 37 °C as I want to determine the effect of DISC1 or NDE1 upon NMDA receptor internalisation. I will analyse the effect of these proteins on the co-localisation between NMDA receptors and the early and late endosomes.

When expressed individually in COS7 cells RAB5 and RAB7 appear as variably sized puncta throughout the cytoplasm and are often highly concentrated in the perinuclear region of the cell (Figure 6.1 A, B). When subjected to a 15 minute incubation at 37^{O}C the localisation of neither RAB5 nor RAB7 is significantly altered (Figure 6.1 C, D)

Under control conditions (i.e. incubation at 4 °C) COS7 cells co-transfected with GluN1, HA-GluN2B and mCherry-RAB5 show no co-localisation between surface-labelled NMDA receptors and RAB5, and moderate co-localisation between NMDA receptors detected post-fixation using the GluN1 antibody (total NMDA receptors) and RAB5, as expected (Figure 6.1 E-H). After incubation at 37 °C the NMDA

receptors are markedly different, appearing as larger structures whether surface/internalised or the total receptor population is detected (Figure 6.1 I). There is also clear co-localisation of the HA-labelled NMDA receptors with RAB5 (Figure 6.1 J-L and see later figures for quantification). This is in agreement with previous studies which showed co-localisation between tac-GluN2B chimeras (containing the first 20 amino acids of the C-terminal tail) and GFP-RAB5 after incubation at 37 °C (Scott et al., 2004).

In cells transfected with GluN1, GluN2B and mCherry RAB7 there is little co-localisation between surface-labelled NMDA receptors and RAB7 under control conditions (Figure 6.1 M-P), although total NMDA receptor staining as detected with the anti-GluN1 antibody post-fixation, exhibits partial co-localisation with the RAB7 signal, as would be expected. After incubation, there remains little co-localisation between the NMDA receptors and RAB7 (Figure 6.1 Q-T), which is in accordance with the published literature.

This optimisation shows that the assay I have developed detects NMDA receptor internalisation along the endocytic pathway to early endosomes/sorting endosomes where RAB5 is located. I will now examine the effects of DISC1 and DISC1-607F upon the co-localisation of RAB5 and RAB7 with NMDA receptors. Because DISC1-607F causes a decrease in surface-expressed NMDA receptors, I will use this assay to try to understand whether the mechanism by which the 607F variant exerts this effect involves endocytosis. Additionally the effects of NDE1, NDE1-131A and NDE1-131E will be studied because NDE1-131E causes decreased NMDA receptor surface expression when compared to NDE1-131A.

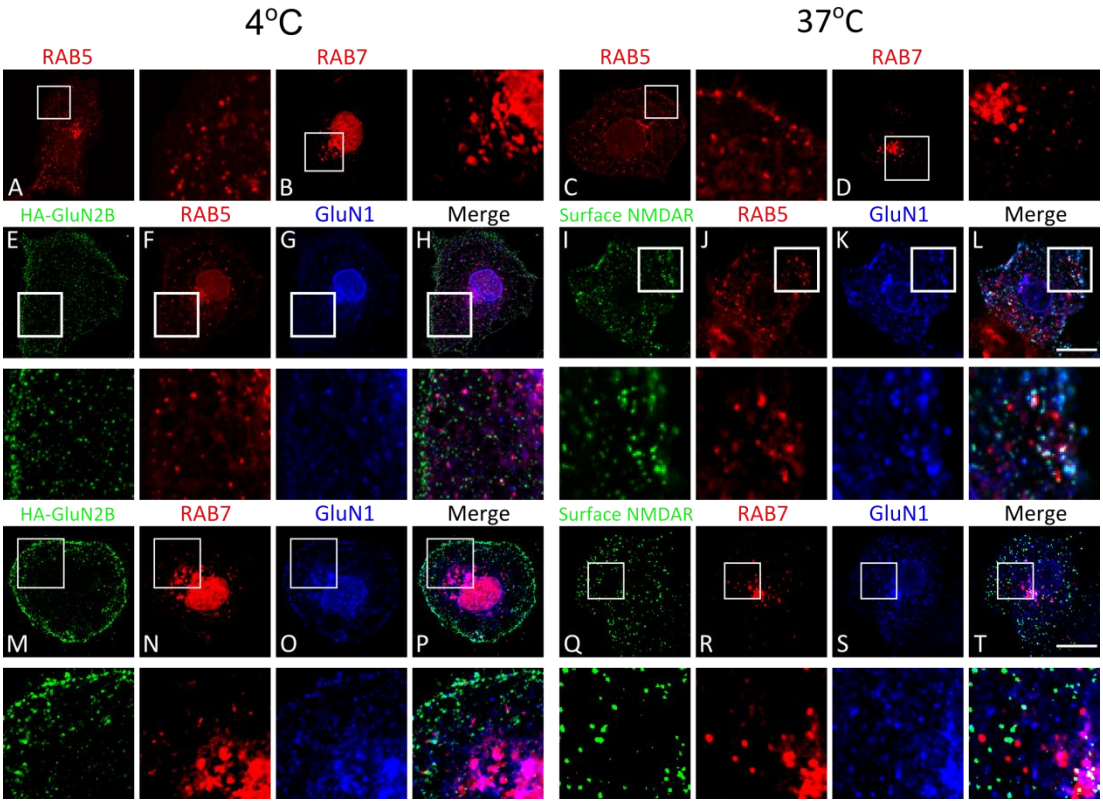

Figure 6.1 Optimisation of recycling assay

mCherry-tagged RAB5 (A) and RAB7-expressing (B) constructs were singly transfected into COS7 cells. C and D show mCherry-RAB5 or mCherry-RAB7 transfected COS7 cells after a 15 minute incubation at 37 °C which allows internalisation of the surface NMDA receptors. Expression of RAB5 and RAB7 is still detectable after incubation.

COS7 cells were also co-transfected with GluN1, HA-GluN2B and mCherry-RAB5. GluN2B was surface labelled at 4 °C and then cells were either incubated at 4 °C for 15 mins (E-F) or at 37 °C (I-L). Incubation at 37 °C caused internalisation of the surface expressed NMDA receptors (I) and increase in RAB5 surface NMDA receptor co-localisation (L).

In addition, COS7 cells were transfected with GluN1, HA-GluN2B and mCherry-RAB7. GluN2B was surface labelled at 4 °C and cells were then incubated at either 4 °C for 15 mins (M-P) or at 37 °C for 15 mins (Q-T). Again the surface-labelled NMDA receptors became internalised (Q) but co-localisation with RAB7 was unchanged following incubation at 37oC. Areas of cells encompassed by white boxes are enlarged to the right in the top row and below in the next two rows.n=3, Scale bars 20 µm. Images G, K, O and S were pseudocolored blue using ImageJ

6.3.2 Quantification of signals

Images of the cells were captured using a Nikon A1R confocal microscope; the same settings (laser power, offset, gain and pinhole size) were used when taking all the images for each experiment. The images for each experiment were taken at the same time to allow for direct comparison between the images. Once the images were acquired they were opened in the imaging programme ImageJ and using the plugin “co-localisation threshold” the image was analysed and the Pearsons coefficient was generated to indicate the level of co-localisation between two of the chosen proteins (e.g. RAB5/surface labelled NMDA receptors or RAB5/DISC1). To ensure only the co-localisation from the cell of interest was measured, a region of interest (ROI) was drawn around the cell and only the co-localisation within that ROI was analysed.

6.3.3 DISC1 co-localises with RAB5- and RAB7-positive structures

I first investigated whether DISC1 co-localises with components of the endocytic pathway, to determine whether any effects upon NMDA receptor endocytosis may be due to direct effects upon the endocytic machinery, upon NMDA receptors, or both. I began by analysing co-localisation of DISC1 and DISC1-607F with RAB5. An association between DISC1 and markers of the early endocytic pathway has not previously been demonstrated. However I found that DISC1 shows partial co-localisation with RAB5 (Figure 6.2 A-C) under control conditions. After incubation at 37^{o}C the level of co-localisation between DISC1 and RAB5 increases slightly (Figure 6.2 D-F). DISC1-607F also shows partial co-localisation with RAB5 under control conditions (Figure 6.2 G-I).This is unchanged by incubation at 37 ^{o}C (Figure 6.2 J-L). One-way ANOVA demonstrated that there was no significant effect of incubation, or of the variant, upon co-localisation between DISC1 or DISC1-607F and RAB5 (Figure 6.2 M). Altogether therefore, these data demonstrate that DISC1 co-localises with a marker of the early endocytic pathway, and that this association with early endosomes appears to be stable and not influenced by the common DISC1 variant 607F.

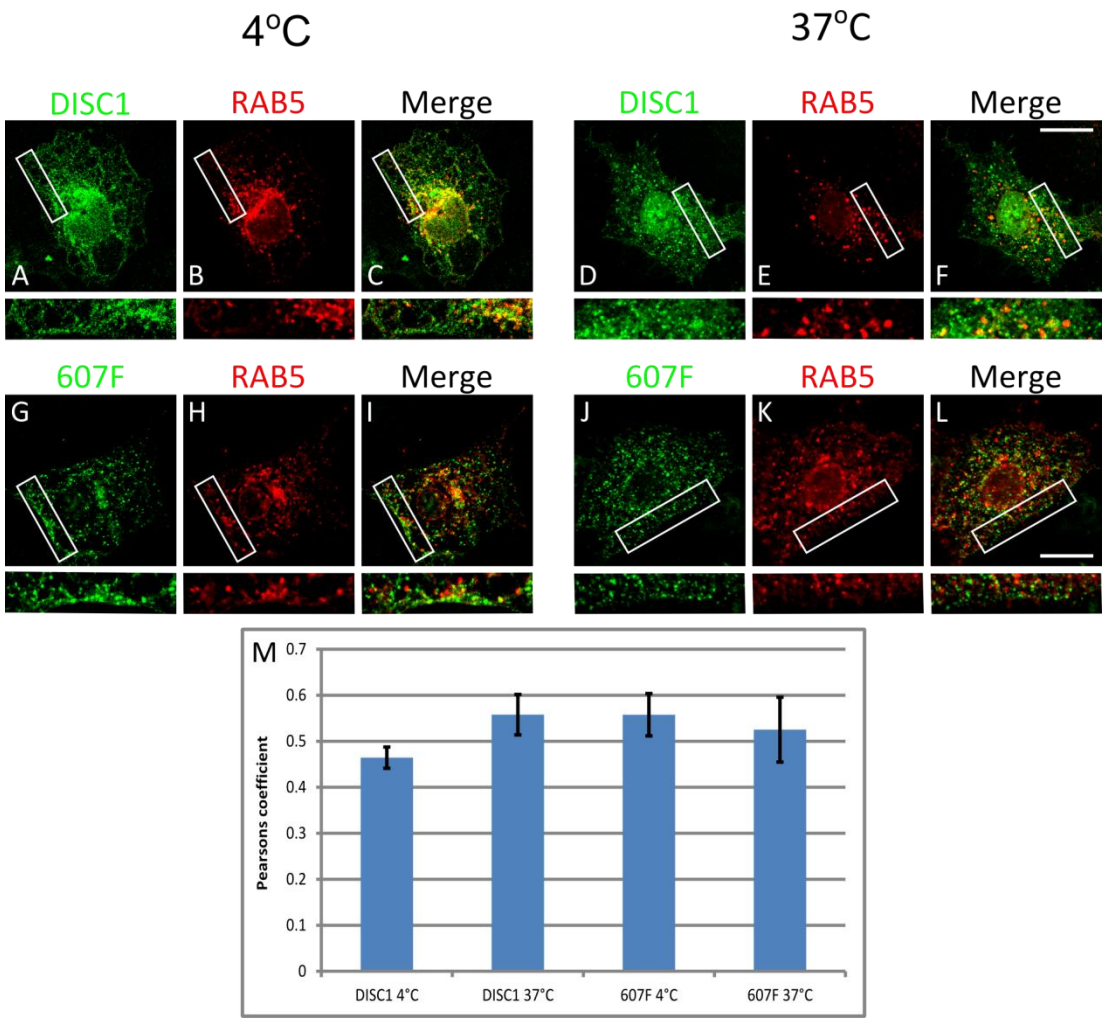

Figure 6.2 Co-localisation of RAB5 and DISC1 or DISC1-607F is unaffected by incubation at 37°C.

COS7 cells co-transfected with plasmid constructs expressing GluN1, HA-GluN2B, mCherry-RAB5 and FLAG-DISC1 were labelled live for surface NMDA receptors and then either maintained at 4 °C or incubated at 37 °C for 15 minutes to allow for receptor internalisation. Cells maintained at 4 °C showed moderate co-localisation between DISC1 (A) and RAB5 (B merge C). After incubation at 37 °C there was no change in co-localisation of DISC1 (D) and RAB5 (E merge F). COS7 cells co-transfected with GluN1, HA-GluN2B, mCherry-RAB5 and FLAG-DISC1-607F were treated in the same way. Cells maintained at 4 °C showed moderate co-localisation between DISC1-607F (G) and RAB5 (H merge I). After incubation at 37 °C there was no change in co-localisation of DISC1-607F (J) and RAB5 (K merge L). Scale 20 µm

Histogram (M) represents Pearsons co-localisation coefficient taken from 20 cells per condition per experiment from three independent experiments, total=60 cells per condition. Error bars represent SEM. Images A, D, G and J were pseudocolored green using ImageJ.

I also analysed co-localisation between RAB7 and DISC1 or DISC1-607F to determine if this occurs and if there is any change after incubation at 37^{o}C. Cells co-expressing DISC1 and RAB7 which were not subjected to incubation showed moderate co-localisation (Figure 6.3 A-C). After incubation there was no change in co-localisation between DISC1 and RAB7 (Figure 6.3 D- F). Similarly cells expressing DISC1-607F and RAB7 under control conditions showed moderate co-localisation (Figure 6.3 G-I). After incubation the level of co-localisation was largely unchanged (Figure 6.3 J-L). Furthermore, comparison of DISC1 co-transfected cells and DISC1-607F co-transfected cells using one-way ANOVA showed no significant difference in RAB7 co-localisation either before or after incubation (Figure 6.3 M).

Altogether these data demonstrate that DISC1 co-localises stably with a proportion of early and late endosomes, and that this association appears to be unaffected by the 607F variant.

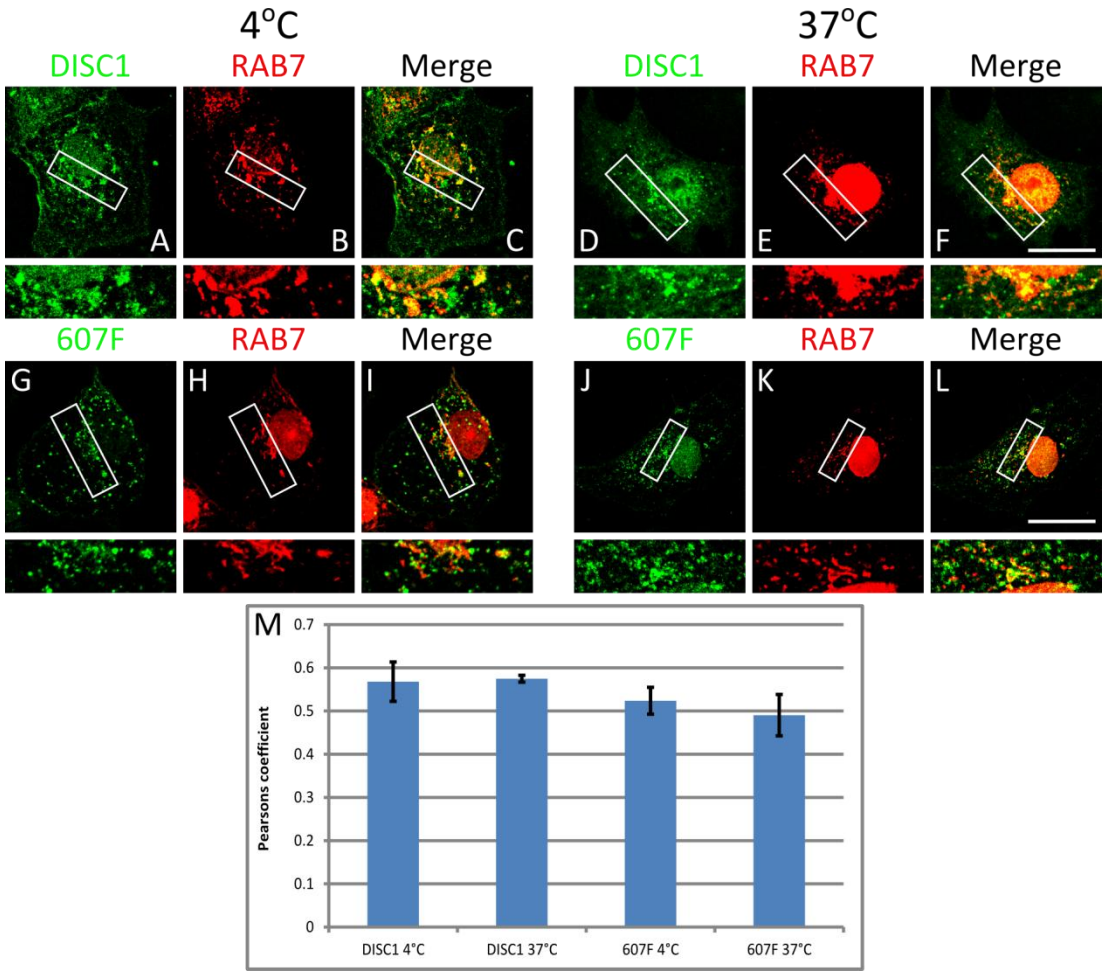

Figure 6.3 RAB7 association with DISC1 and DISC1-L607F

COS7 cells were transfected with GluN1, HA-GluN2B, mCherry-RAB7 and either FLAG-DISC1 or FLAG-DISC1-607F. The cells were stained live for surface NMDA receptors and then either maintained at 4 °C or incubated at 37 °C for 15 minutes to allow for internalisation of the NMDA receptors. Cells were then stained for total GluN1 and DISC1 and imaged. Non-incubated cells show some co-localisation of DISC1 (A) and RAB7 (B merge C). This was unaltered after a 15 minute incubation at 37 °C (D-F). Similarly in non-incubated cells DISC1-607F (G) co-localised with RAB7 (H merge I). After incubation DISC1-607F (J) exhibits similar co-localisation with RAB7 (K merge L). Scale 20 µm.
Histogram (M) represents the Pearsons co-efficient of co-localisation between DISC1 and RAB7 and DISC1-607F and RAB7 before and after incubation at 37 °C. 20 cells per condition from three independent experiments, total=60 cells per condition. Error bars represent SEM. Images A, D, G and J were pseudocolored green using ImageJ

6.3.4 NDE1 co-localises with RAB5- and RAB7-positive structures

Next, co-localisation of RAB5 with NDE1, NDE1-131A or NDE1-131E was measured to determine whether 1) NDE1 associates with early endocytic vesicles 2) any co-localisation with RAB5 is influenced by the phosphosite mutations, and 3) there is any effect of incubating the cells at 37 °C. Under basal conditions, NDE1 staining appeared as described in chapter 4. It is punctate in appearance and distributed throughout the cytoplasm (Figure 6.4 A). RAB5 (Figure 6.4 B) appeared as described in 6.3.1 and there is moderate co-localisation between the two proteins (Figure 6.4 C). After incubation the staining pattern of NDE1 appears largely unchanged (Figure 6.4 D and E). Similarly, under basal conditions there is moderate co-localisation between RAB5 and NDE1-131A (Figure 6.4 G-I) or NDE1-131E (Figure 6.4 M-O). After incubation there is a small decrease in the co-localisation between; RAB5 and NDE1 (Figure 6.4 D-F), RAB5 and NDE1-131A (Figure 6.4 J-L) and RAB5 and NDE1-131E (Figure 6.4 P-R). None of these effects are significant however (Figure 6.4 P).

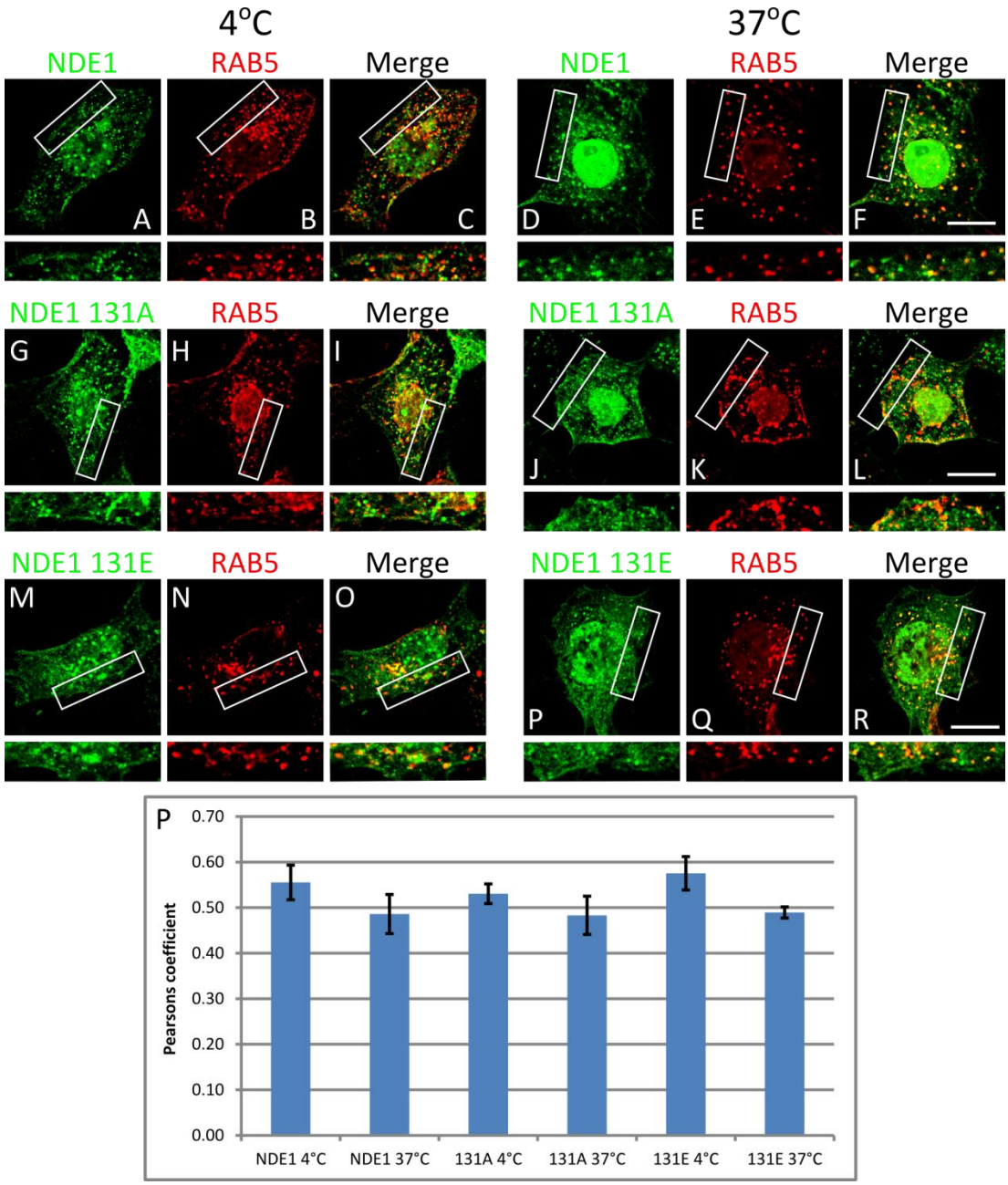

Figure 6.4 RAB5 co-localises with NDE1

COS7 cells were transfected with GluN1, HA-GluN2B, mCherry-RAB5 and either V5-NDE1, V5-NDE1-131A or V5-NDE1-131E. The cells were maintained at 4°C for 15 minutes or incubated at 37°C to promote receptor endocytosis. Cells were then fixed and stained for total NDE1 or phosphomutant NDE1. NDE1 (A) co-localises with RAB5 (B merge C) and this decreases slightly after incubation (D-F). NDE1-131A (G) co-localises with RAB5 (H merge I) under basal conditions. Incubation slightly decreases the co-localisation (J-L). Similarly NDE1-131E (M) co-localises with RAB5 (N merge O). Incubation slightly decreases this co-localisation (P-R). Scale 20 µm

Histogram (S) represents Pearsons co-efficient taken from 20 images per condition per experiment from 3 independent experiments, total=60 cells per condition. Error bars represent SEM. Images A, D G, J, M and P were pseudocolored green using ImageJ

Co-localisation between RAB7 and NDE1, NDE1-T131A and NDE1-T131E was then investigated to determine whether NDE1 may associate with late endocytic vesicles, and to find out if there are any effects of the phosphosite mutants, or of incubating the cells at 37 °C. Cells co-expressing NDE1, under control conditions gave a robust staining pattern for NDE1 (Figure 6.5 A), strong RAB7 staining (Figure 6.5 B) and there was moderate co-localisation between the two proteins (Figure 6.5 C). After incubation there was no change in the co-localisation of NDE1 and RAB7 (Figure 6.5 D-E). Similarly in cells co-expressing NDE1-131A there was robust NDE1-131A (Figure 6.5 G) and RAB7 signal (Figure 6.5 H) with moderate co-localisation between the two proteins (Figure 6.5 I). After incubation there was no change in the amount of co-localisation between NDE1-131A and RAB7 (Figure 6.5 J-L). Cells co-expressing NDE1-131E under control conditions gave robust staining of NDE1-131E (Figure 6.5 M) and RAB7 (Figure 6.5 N) with moderate co-localisation (Figure 6.5 O). Analysis of NDE1/RAB7 co-localisation using one-way ANOVA found no significant effects of the phosphosite mutants, or of incubation at 37^{o}C, thus this association appears to be stable.

As observed for DISC1, it is apparent that NDE1 co-localises with early and late endosomes, and that this association is unaffected by internalisation events, or by the phosphosite mutants.

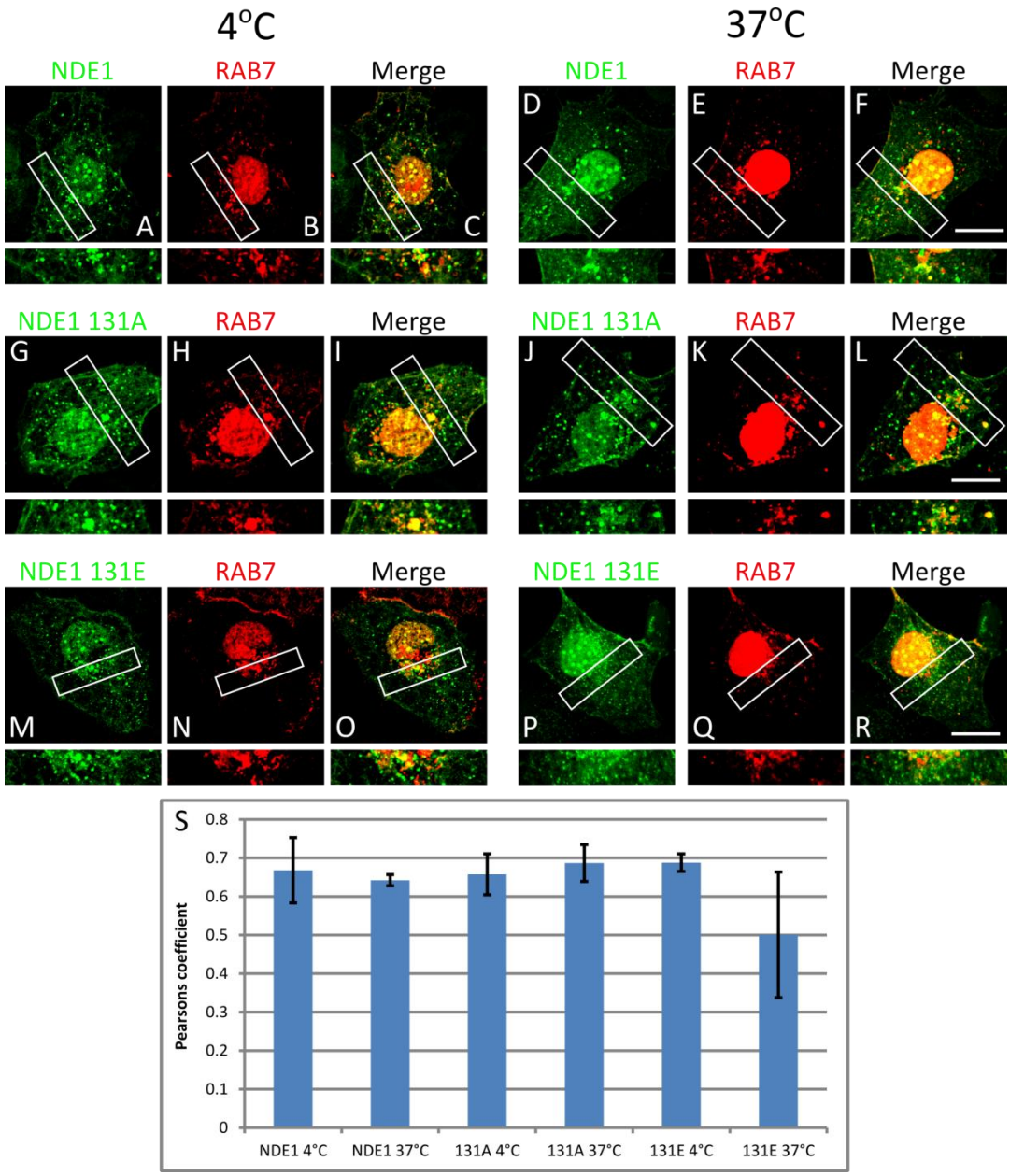

Figure 6.5 NDE1/RAB7 co-localisation

COS7 cells were transfected with GluN1, HA-GluN2B and mCherry-RAB7, and either empty vector (EV), V5-NDE1, V5-NDE1-131A or V5-NDE1-131E. GluN2B was surface-labelled at 4 °C and either incubated at 37 °C for 15 minutes to allow for receptor internalisation, or at 4 °C for 15 minutes as a control (surface NMDA receptor labelling is shown in Figure 6.1). NDE1 (A) co-localises with RAB7 (B,C) in control cells and this is unchanged after incubation (D-F). NDE1-131A (G) co-localises with RAB7 (H,I) under control conditions and this is unchanged after incubation (J-L). NDE1-131E (M) co-localises with RAB7 (N,O) under control conditions and this is unchanged after incubation (P-R). Scale 20 µm.

Histogram (S) shows Pearsons co-efficient of RAB7 and NDE1 or phosphomutant NDE1 co-localisation. 20 images were taken per condition in 3 independent experiments, total=60 cells per condition. Error bars represent SEM. Images A, D, G, J, M and P were pseudocolored green using ImageJ

6.3.5 DISC1-607F may inhibit internalisation of surface-expressed NMDA receptors

I next wanted to investigate whether DISC1 and/or NDE1 influence NMDA receptor internalisation, beginning with DISC1. COS7 cells were transfected with plasmid constructs expressing GluN1, HA-GluN2B, mCherry-RAB5 and either DISC1, DISC1-607F or corresponding empty vector (EV). Cells were stained live for surface NMDA receptors using the anti-HA antibody, and then incubated for 15 minutes at either 4 °C or 37^{o}C. In cells co-expressing EV there is little co-localisation between surface-labelled NMDA receptors and RAB5 (Figure 6.6 A-C). After incubation the surface-labelled NMDA receptors become internalised and exhibit some co-localisation with RAB5 (Figure 6.6 D-F). This was to be expected as it has been found previously that upon internalisation NMDA receptors co-localise with RAB5 (Scott et al., 2004). DISC1 transfected cells similarly show little co-localisation between surface-labelled NMDA receptors and RAB5, compared to that observed in EV-transfected cells under basal conditions (Figure 6.6 G-I). After incubation there is an increase in co-localisation of surface-labelled NMDA receptors and RAB5 (Figure 6.6 J-L). DISC1-607F expressing cells similarly show limited co-localisation between surface-labelled NMDA receptors and RAB5 (Figure 6.6 M-O), equivalent to that of EV or DISC1-transfected cells under basal conditions. But in this case, following incubation, surface-labelled NMDA receptor co-localisation with RAB5 remains approximately the same, indicating a possible inhibitory effect of the 607F variant (Figure 6.6 P-R).

One-way ANOVA indicates a significant effect of incubation at 37^{o}C upon NMDA receptor/RAB5 co-localisation (Figure 6.6 S, $p=0.02$). Post-hoc testing using Bonferroni's Pairwise Comparison Test revealed that this is due to increased surface-labelled NMDA receptor/RAB5 co-localisation in EV-transfected cells following incubation ($p<0.05$), an effect that is not seen in cells transfected with DISC1-607F. In cells transfected with DISC1, there is increased receptor/RAB5 co-localisation following incubation, but this does not survive correction for multiple

testing ($p<0.05$ without correction for multiple comparisions, $p>0.05$ following correction). These data therefore indicate that DISC1-607F likely inhibits NMDA receptor recruitment to RAB5-positive structures following incubation at 37^{o}C. Alternatively, it is possible that in the presence of DISC1-607F, NMDA receptors proceed more quickly along the endocytic pathway, with the result that no effect is detectable upon receptor/RAB5 co-localisation at the time-point used.

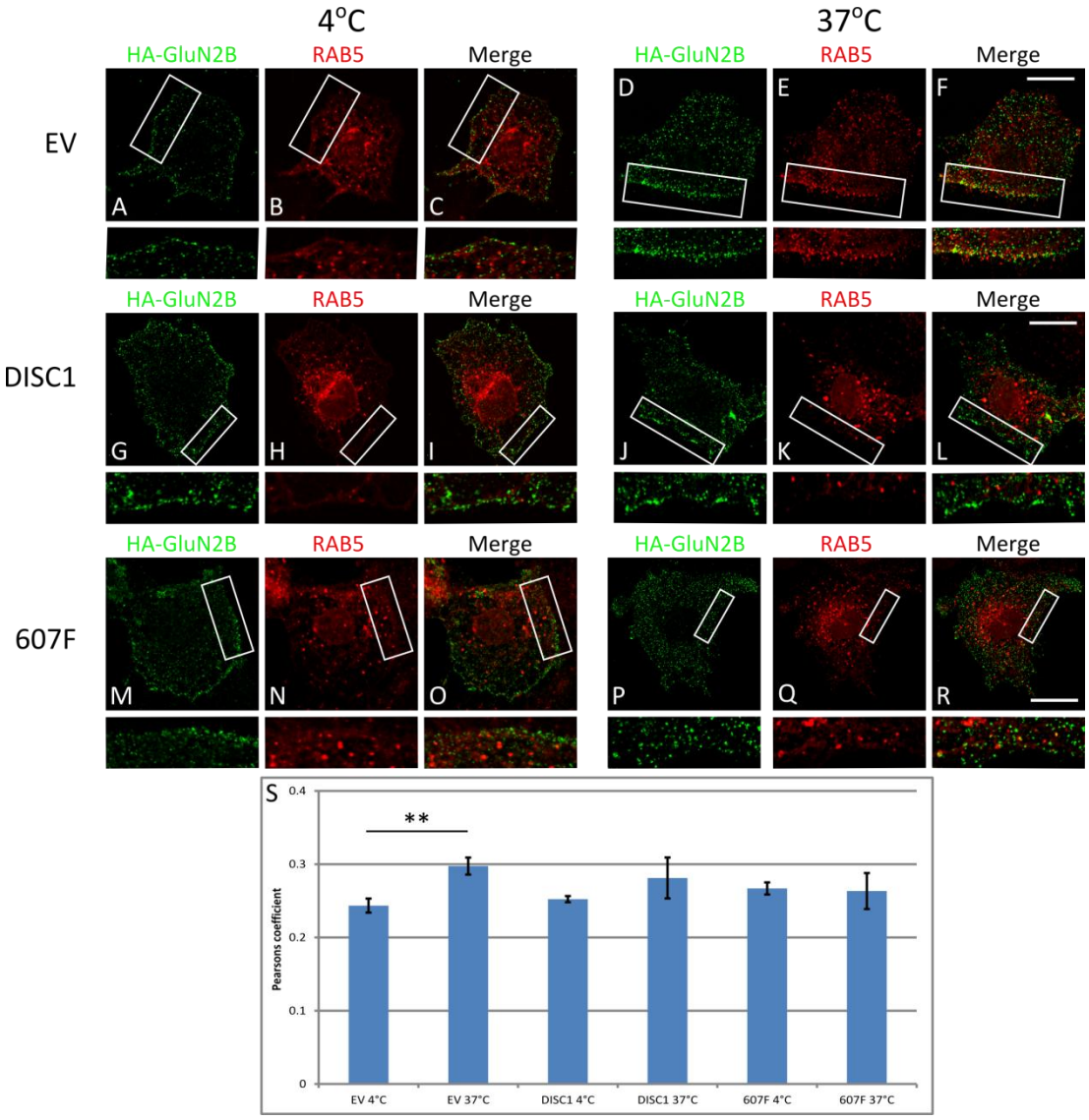

Figure 6.6 DISC1-607F blocks NMDA receptor recruitment to RAB5-positive structures following incubation at 37°C.

COS7 cells were transfected with GluN1, HA-GluN2B and mCherry-RAB5, and either empty vector (EV), FLAG-DISC1 or FLAG-DISC1-607F. Cells were surface labelled at 4 °C and either incubated at 37 °C for 15 minutes to allow for receptor internalisation or at 4 °C for 15 minutes as a control. In EV-transfected cells there was a significant increase in co-localisation after incubation (A-F). DISC1 co-transfected cells showed no change in surface NMDA receptor co-localisation with RAB5 (G-L) after incubation at 37°C. DISC1-607F co-expressing cells also do not exhibit a change in surface NMDA receptor co-localisation with RAB5 after incubation at 37°C (M-R). Scale bars 20 µm.

Histogram (S) represents the Pearsons co-efficient of co-localisation of surface labelled NMDA receptors and RAB5. Data represents 20 images per condition from three independent experiments, total=60 cells per condition. ** P<0.01. Error bars represent SEM

To examine this further, I next determined if DISC1 or DISC1-607F have any effect on NMDA receptor co-localisation with RAB7, a marker of late endosomes, where it is possible I would detect increased NMDA receptor localisation if their transit along the endocytic pathway has been accelerated. COS7 cells were transfected with plasmid constructs expressing GluN1, HA-GluN2B, RAB7 and either EV, DISC1 or DISC1-607F and treated as described in 6.3.1. EV-transfected cells show little co-localisation between surface labelled NMDA receptors and RAB7 before incubation (Figure 6.7 A-C), or after incubation at 37^{o}C (Figure 6.7 D-F). DISC1-expressing cells under control conditions also show limited co-localisation between surface-labelled NMDA receptors and RAB7 before (Figure 6.7 G-I) or after incubation (Figure 6.7 J-L). Similarly, DISC1-607F-expressing cells show limited co-localisation between surface-labelled NMDA receptors and RAB7 before (Figure 6.7 M-O) or after incubation (Figure 6.7 P-R).

One-way ANOVA detected no effects of DISC1, the DISC1 607F variant or incubation at 37^{o}C (Figure 6.7 S). It therefore appears that the effect of the 607F variant is most likely inhibition of NMDA receptor internalisation, rather than promotion of faster receptor movement along the degradation pathway.

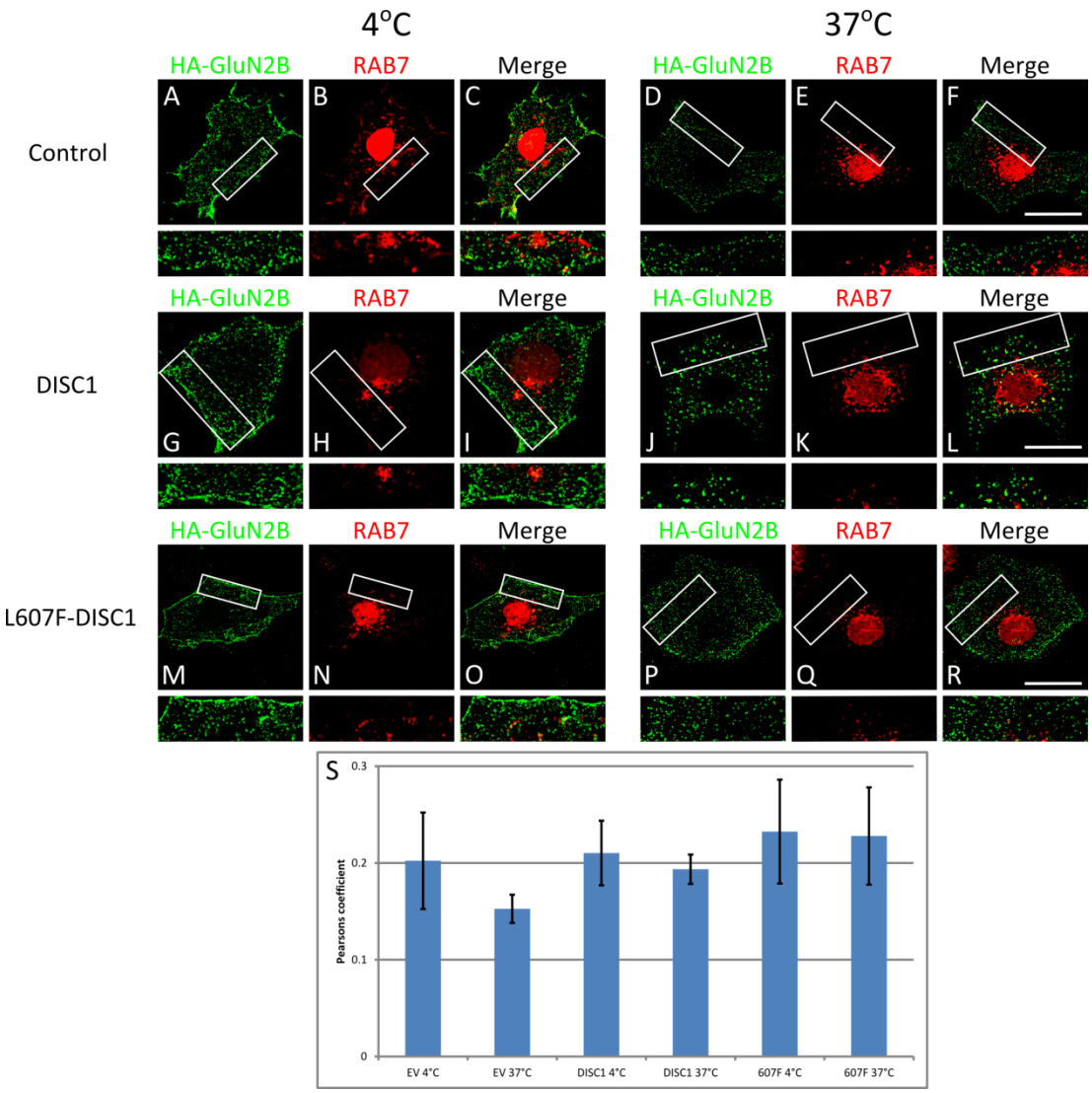

Figure 6.7 Surface-labelled NMDA receptor non co-localisation with RAB7 is unaffected by DISC1.

COS7 cells were transfected with GluN1, HA-GluN2B and mCherry-RAB7, and either EV, FLAG-DISC1 or FLAG-DISC1-607F. The cells were surface labelled at 4 °C and either incubated at 37 °C for 15 minutes to allow for receptor internalisation or at 4 °C for 15 minutes as a control. In EV (A-F), DISC1 (G-L) or DISC1-607F (M-R) transfected cells there was no change in surface NMDA receptor co-localisation with RAB7 after incubation. Scale bar 20 µm

Histogram (S) shows Pearsons co-efficient of co-localisation taken from 20 cells per condition from three independent experiments, total=60 cells per condition. Error bars represent SEM

6.3.6 DISC1-L607F co-localises more strongly with surface NMDA receptors than DISC1, prior to internalisation

I next measured DISC1 and DISC1-607F co-localisation with surface labelled NMDA receptors. An association of DISC1 and the GluN1 subunit has been previously shown (S.Mackie unpublished) however co-localisation at the surface of the cell had not yet been investigated. My studies now show there is co-localisation between DISC1 and surface labelled NMDA receptors at 4 °C (Figure 6.8 A-C). After incubation at 37 °C there is no change in co-localisation between DISC1 and surface labelled NMDA receptors (Figure 6.8 D-F). Similarly, cells co-transfected with DISC1-607F show no significant difference in co-localisation between DISC1-607F and surface labelled NMDA receptors after incubation at 37 °C (Figure 6.8 G-L). There is, however, increased NMDA receptor/DISC1 co-localisation at 4^{o}C in the presence of the 607F variant.

One-way ANOVA confirmed a significant effect between the amount of co-localisation between DISC1 and surface expressed NMDA receptors and DISC1-607F and surface expressed NDMA receptors ($p=0.02$, Figure 6.8 M) and post-hoc pairwise Bonferroni testing revealed a significant difference between the levels of co-localisation of DISC1/surface labelled NMDA receptors and DISC1-607F/surface labelled NMDA receptors at 4 °C ($p<0.01$). Altogether therefore, these data demonstrate that DISC1 co-localises with surface labelled NMDA receptors, and that this association appears to be stable following internalisation. Also there appears to be a more robust co-localisation between surface labelled NMDA receptors with DISC1-607F at the surface of cells.

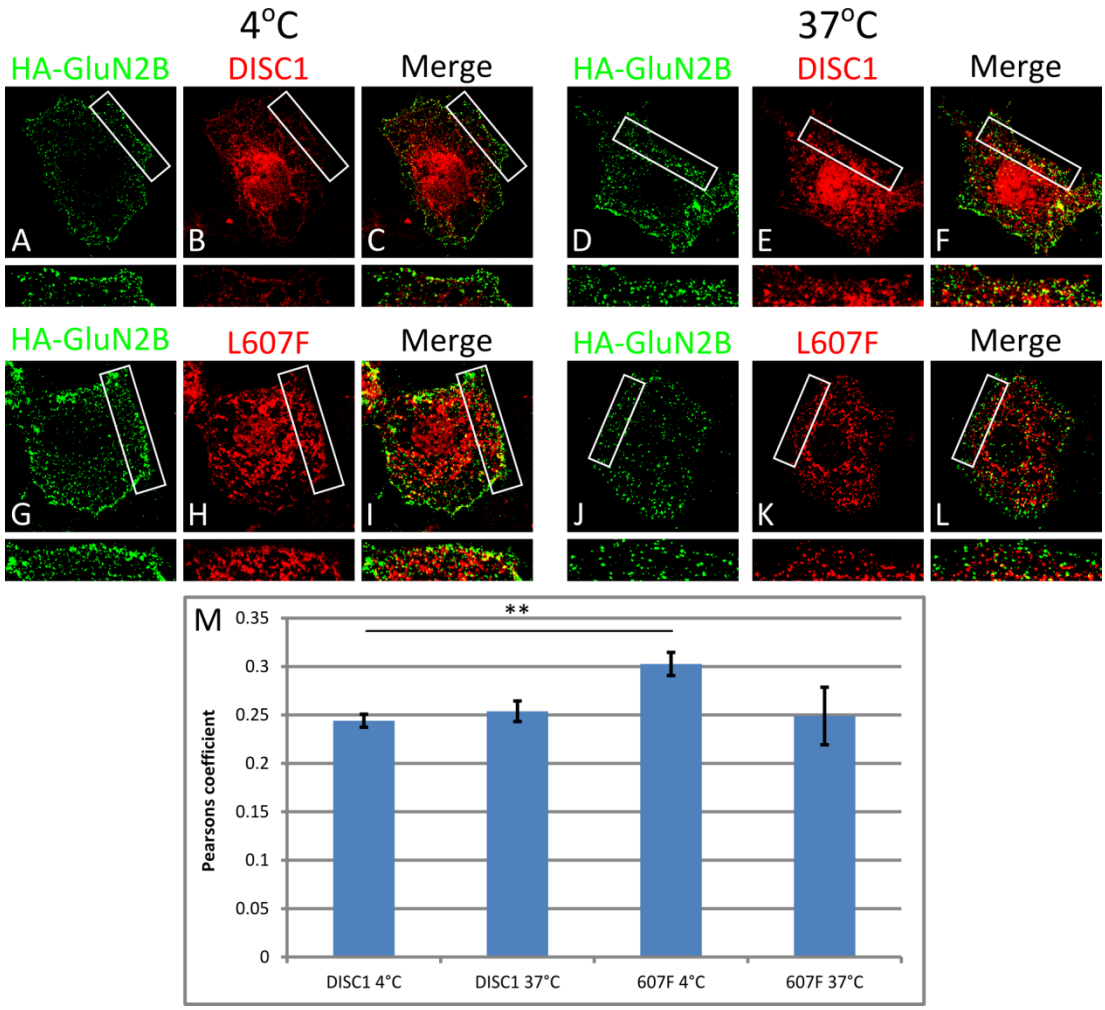

Figure 6.8 The DISC1 607F variant increases DISC1 association with surface NMDA receptors

COS7 cells co-transfected with plasmid constructs expressing GluN1, HA-GluN2B, mCherry-RAB5 and FLAG-DISC1 were labelled live for surface NMDA receptors and then either maintained at 4 °C or incubated at 37 °C for 15 minutes to allow for receptor internalisation. Cells maintained at 4 °C showed some co-localisation between DISC1 (A) and surface labelled NMDA receptors (B merge C). After incubation at 37 °C there was no change in co-localisation of DISC1 (D) and surface labelled NMDA receptors (E merge F). COS7 cells co-transfected with GluN1, HA-GluN2B, mCherry-RAB5 and FLAG-DISC1-607F were treated in the same way. Cells maintained at 4 °C showed moderate co-localisation between DISC1-607F (G) and surface labelled NMDA receptors (H merge I). After incubation at 37 °C there was a significant decrease in co-localisation between DISC1-607F (J) and surface labelled NMDA receptors (K merge L). Scale 20 µm

Histogram (M) represents Pearsons co-localisation coefficient taken from 20 cells per condition per experiment from three independent experiments, total=60 cells per condition.** P<0.01 . Error bars represent SEM. Images B, E, H and K were pseudocolored red using ImageJ.

6.3.7 Investigating the effects of NDE1 and NDE1 phosphositemutants upon NMDA receptor internalisation

COS7 cells were transfected with GluN1, HA-GluN2B, mCherry-RAB5 and either EV, NDE1, NDE1-131A or NDE1-131E. In EV-transfected cells, under control conditions, there is strong NMDA receptor surface-labelled expression and RAB5 expression but with little co-localisation apparent (Figure 6.9A-C). After incubation, the surface-labelled NMDA receptors become internalised and there is substantial co-localisation of HA and RAB5 signals as expected (Figure 6.9 D-F). NDE1-transfected cells, under control conditions, exhibit strong surface-labelled NMDA receptor expression and RAB5 expression (Figure 6.9 G-I), but weak co-localisation between surface-labelled NMDA receptors and RAB5, which increases following incubation at 37^{O}C (Figure 6.9 J-L). NDE1-131A and NDE1-131E co-transfected cells similarly show limited co-localisation between surface-labelled NMDA receptors and RAB5 (M-O, S-U, respectively) that increases following incubation at 37^{O}C (Figure 6.9 P-R, V-X, respectively). Taken together this data shows that the expected increase in surface-labelled NMDA receptors with RAB5 occurs after incubation at 37 °C. Co-transfection with NDE1 slightly increases co-localisation of surface labelled NMDA receptors with RAB5 under control conditions, but does not greatly affect the increase in co-localisation of surface labelled NMDA receptors with RAB5 observed after incubation. Phosphomutants NDE1-131A and NDE1-131E have no effect on the increase in surface labelled-NMDA receptor co-localisation with RAB5 after incubation.

Analysis of the data using one-way ANOVA indicated an overall significant effect on the co-localisation of NMDA receptors and RAB5 after incubation at 37 °C (Figure 6.9 Y, $p=0.0004$), which post-hoc Bonferroni pairwise tests revealed to be due to increased NMDA receptor/RAB5 co-localisation after incubation at 37^{O}C in the presence of EV ($p<0.01$), NDE1-131A ($p<0.01$) or NDE1-131E ($p<0.05$). The same trend was apparent in NDE1-transfected cells although this did not reach significance ($p<0.05$ without correction for multiple comparisons, $p>0.05$ following

correction). This is most likely because of the slightly higher baseline co-localisation between surface-labelled NMDA receptors and RAB5 in NDE1-expressing cells, although this difference is only significant prior to correction for multiple testing (co-localisation in EV vs NDE1-transfected cells, $p<0.05$ without correction for multiple comparisons, $p>0.05$ following correction (Figure 6.9 Y). I therefore speculate that NDE1 may weakly promote initial association of NMDA receptors with the endocytic machinery, even prior to onset of internalisation.

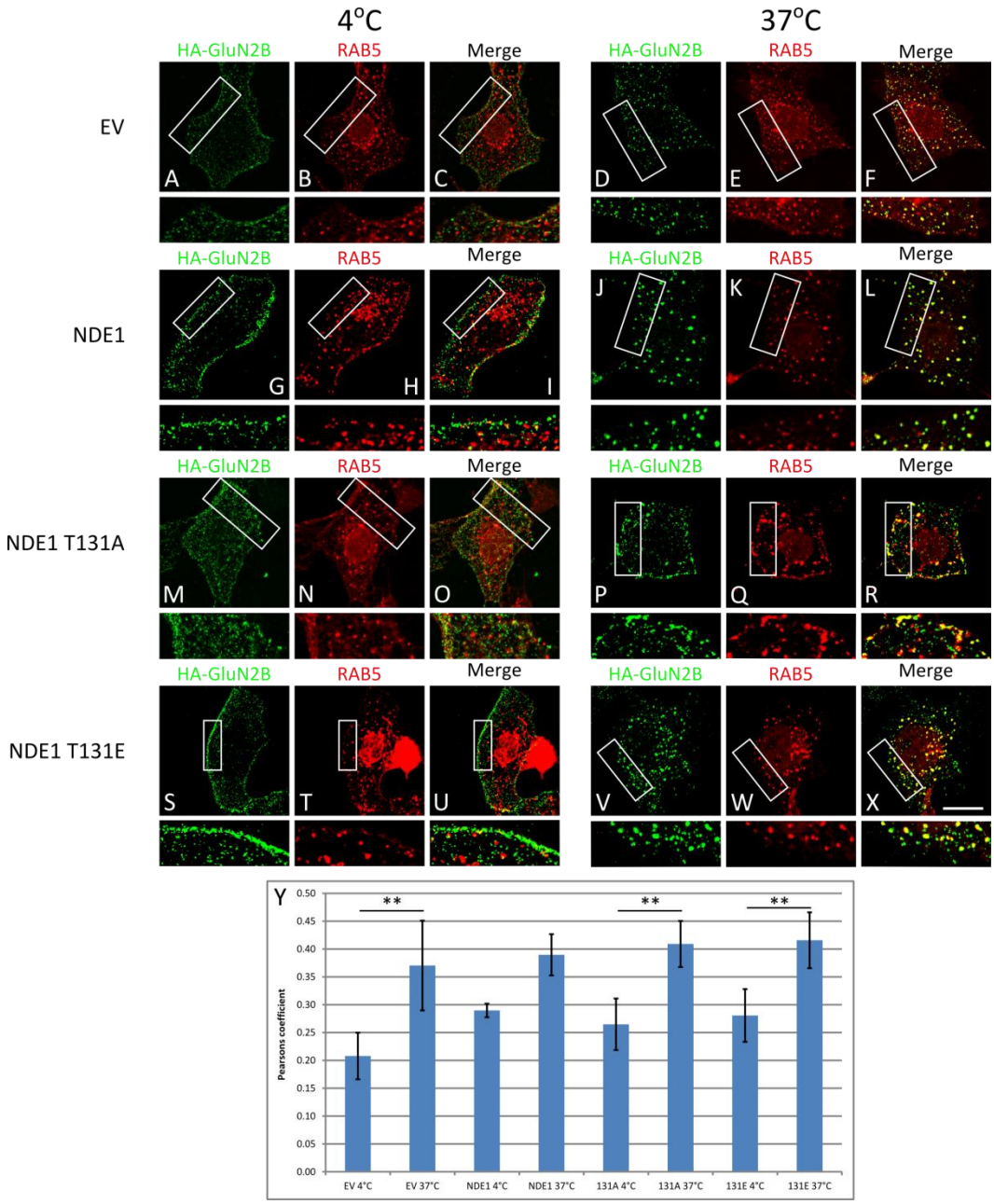

Figure 6.9 RAB5 co-localisation with surface NMDA receptors is unaltered by the presence of NDE1 or NDE1 phospho-mutants.

COS7 cells were transfected with GluN1, HA-GluN2B and mCherry-RAB5, and either empty vector (EV) (A-F), V5-NDE1 (G-L), V5-NDE1-131A (M-R) or V5-NDE1-131E (S-X). The cells were surface-labelled at 4 °C and either incubated at 37 °C for 15 minutes to allow for receptor internalisation or at 4 °C for 15 minutes as a control. In all transfected cells there was an increase in surface-labelled NMDA receptor co-localisation with RAB5 after incubation. Scale bars 20 µm.

Histogram (Y) represents Pearsons co-efficient for RAB5 surface NMDA receptor co-localisation. Values were generated from 20 images per condition from three independent experiments, total=60 cells per condition. **p<0.01, error bars represent SEM.

I next examined whether there is any effect of NDE1 expression upon NMDA receptor association with RAB7-positive late endosomes. COS7 cells were transfected with GluN1, HA-GluN2B, mCherry-RAB7 and either EV, V5-NDE1, V5-NDE1-131A or V5-NDE1-131E. There was little co-localisation between surface NMDA receptors and RAB7 before or after incubation at 37^{o}C (Figure 6.10 A-F). Co-expression of V5-NDE1, either wild-type or mutant, slightly increased surface-labelled NMDA receptor/RAB7 co-localisation (Figure 6.10 G-X) After incubation at 37^{o}C to allow receptor internalisation there was no change in receptor/RAB7 co-localisation, and this was unaffected by co-expression of V5-NDE1, either wild-type or mutant (Figure 6.10 Y).

Analysis using one-way ANOVA found that there is no significant effect of NDE1 or the phosphosite mutants upon surface-labelled NMDA receptor non co-localisation with RAB7-positive structures.

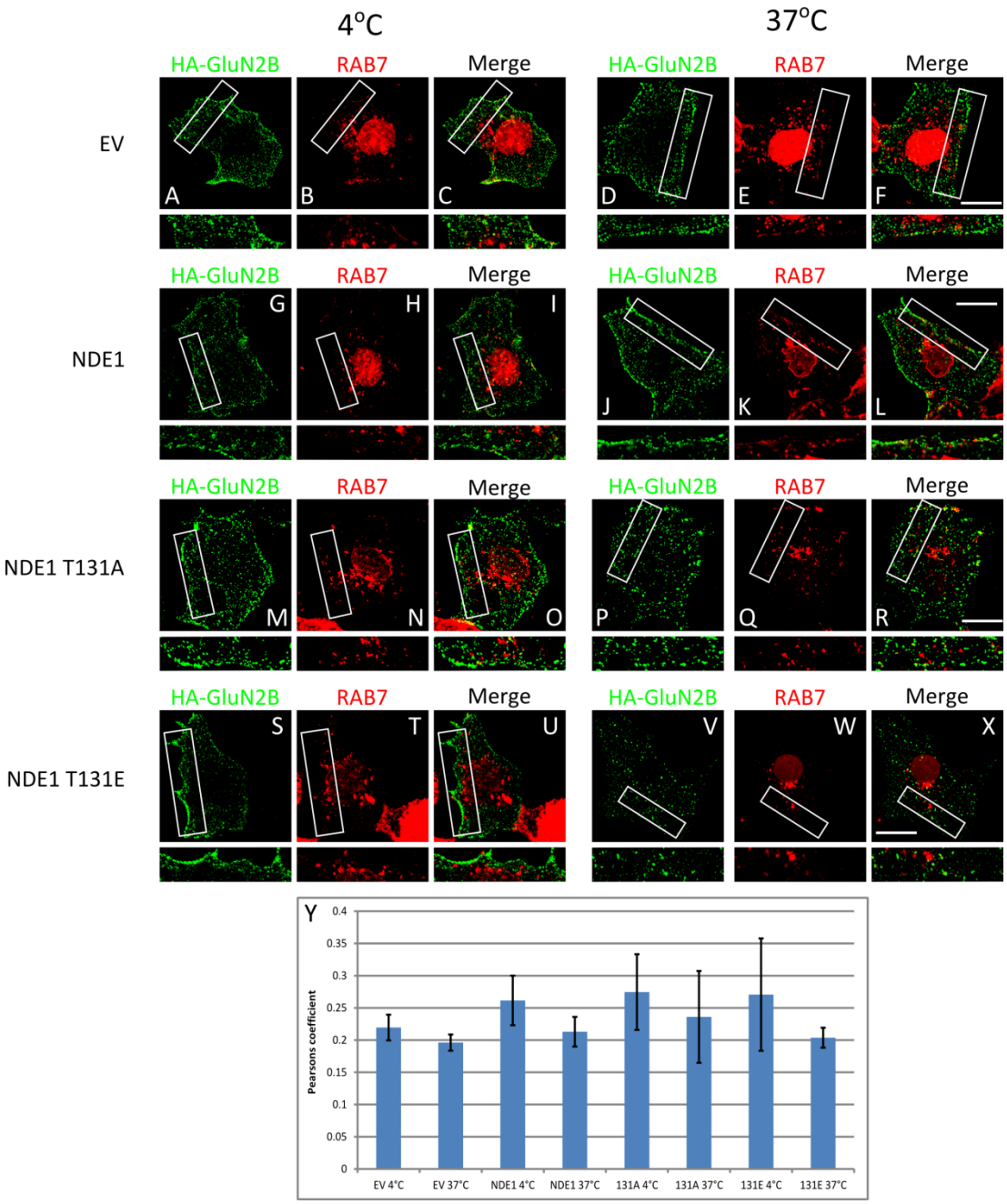

Figure 6.10 RAB7 co-localisation with surface-labelled NMDA receptors is not changed by the presence of NDE1 or phosphosite mutants

COS7 cells were transfected with GluN1, HA-GluN2B and mCherry-RAB7, and either empty vector (EV) (A-F), NDE1 (G-L), NDE1-131A (M-R) or NDE1-131E (S-X). The cells were surface labelled at 4 °C and either incubated at 37 °C for 15 minutes to allow for receptor internalisation or at 4 °C for 15 minutes as a control. Scale bar 20 µm.

Histogram (Y) represents Pearsons co-efficient for co-localisation between surface NMDA receptor and RAB7. 20 images per condition in three independent experiments, total=60 cells per condition. Error bars represent SEM.

6.3.8 NDE1 and surface labelled NMDA receptors

In addition to the measurement of NDE1 co-localisation with RAB5, I measured co-localisation between surface labelled NMDA receptors and NDE1 or NDE1-131A or NDE1-131E. There was co-localisation between NDE1 and surface labelled NMDA receptors (Figure 6.11 A-C) when incubated at 4 °C. After incubation at 37 °C to allow receptor internalisation, there was a decrease in co-localisation between NDE1 and surface labelled NMDA receptors (Figure 6.11 D-F). Cells co-transfected with NDE1-131A and NDE1-131E had similar levels of co-localisation to NDE1 transfected cells when incubated at 4^{o}C (Figure 6.11 G-I and M-O respectively). Similarly after incubation at 37^{o}C there was a decrease in co-localisation between NDE1-131A and surface labelled NMDA receptors Figure 6.11 J-L) and NDE1-131E and surface labelled NMDA receptors (Figure 6.11 P-R).

One way ANOVA revealed a significant effect of incubation at 37^{o}C upon receptor/NDE1 co-localisation ($p=0.007$). Post-hoc pairwise Bonferroni testing revealed this effect is due to a significant decrease in NMDA receptor/NDE1 and NMDA receptor/NDE1-131A co-localisation following internalisation ($p<0.05$ for both comparisons). The decrease in NMDA receptor/NDE1-131E co-localisation did not survive correction for multiple testing ($p<0.05$ prior to multiple comparisons test, $p>0.05$ following the test), but the trend is the same.

This decrease in NMDA receptor/NDE1 co-localisation following receptor internalisation is consistent with my earlier suggestion that NDE1 may promote receptor association with the endocytic machinery prior to onset of internalisation; if NDE1 promotes the association with the endocytic machinery, it may then dissociate from the receptor once internalisation has taken place, although we might expect that the association would be maintained in order to promote dynein-mediated retrograde endosome movement.

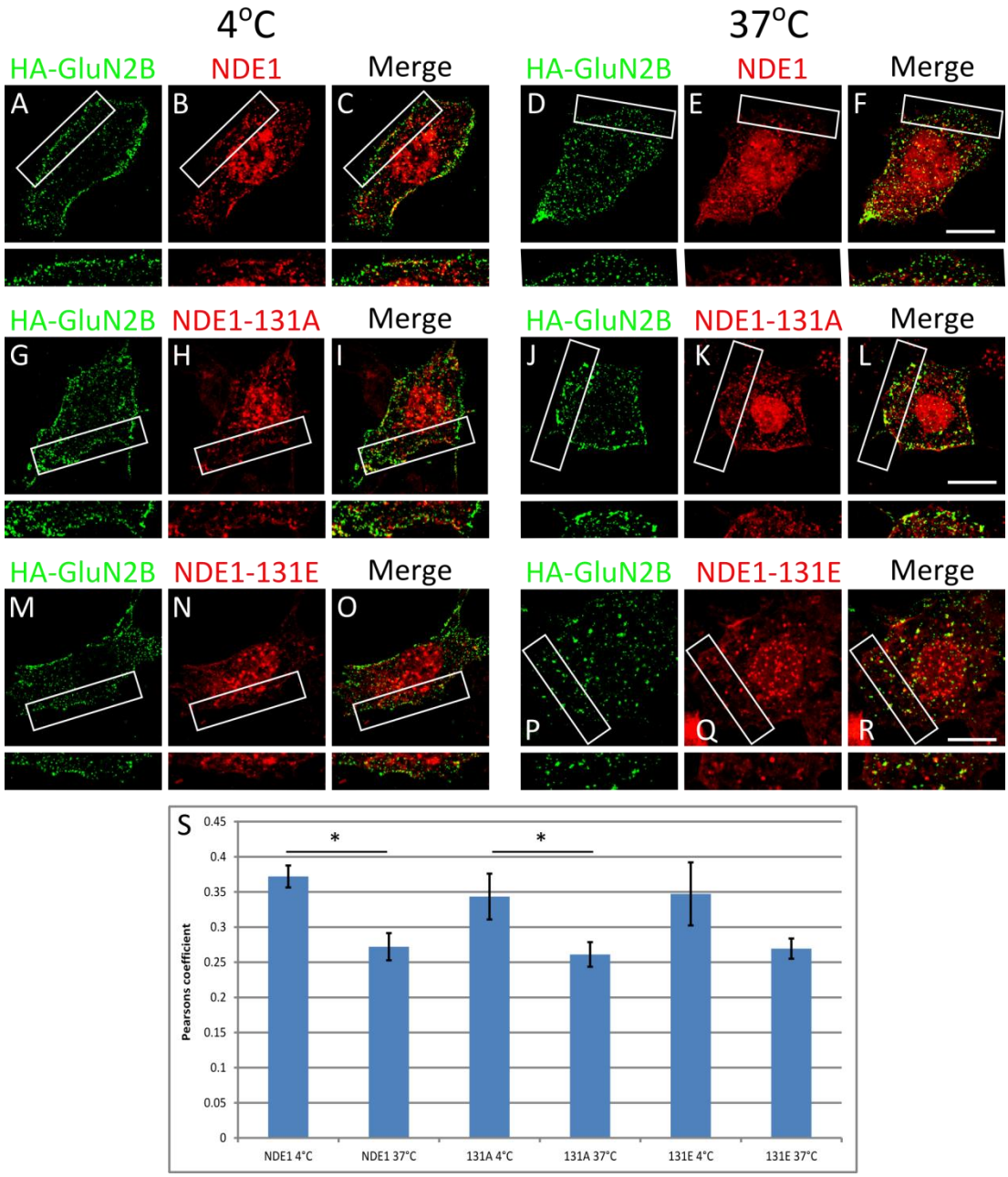

Figure 6.11 Incubation at 37°C decreases surface-labelled NMDA receptor and NDE1 co-localisation

COS7 cells were transfected with GluN1, HA-GluN2B and mCherry-RAB5, and either V5-NDE1 (A-F), V5-NDE1-131A (G-L) or V5-NDE1-131E (M-R). The cells were surface-labelled at 4 °C and either incubated at 37 °C for 15 minutes to allow for receptor internalisation or at 4 °C for 15 minutes as a control. In all transfected cells there was a decrease in surface-labelled NMDA receptor co-localisation with V5-NDE1, V5-NDE1-131A and V5-NDE1-131E after incubation. Scale bars 20 µm.

Histogram (S) represents Pearsons co-efficient for V5-NDE1 surface NMDA receptor co-localisation. Values were generated from 20 images per condition from three independent experiments, total=60 cells per condition. *p<0.05, error bars represents SEM. Images B, E, H, K, N and Q were pseudocolored red using ImageJ.

6.4 Discussion

NMDA receptor endocytosis is a highly regulated process, the exact mechanisms of which are still unknown. The results from this chapter suggest an involvement for both DISC1 and NDE1, potentially shining light on new areas of the mechanism.

I analysed co-localisation between DISC1 or DISC1-607F and RAB5 or RAB7, markers of endocytic vesicles/early endosomes and late endosomes, respectively. A proportion of DISC1 and RAB5 co-localise under control conditions, suggesting that DISC1 co-localises with endocytic vesicles/early endosomes, although additional work is required before firm conclusions can be made about this. Such work could include subcellular fractionation to isolate endosomes, additional co-localisation work in other cell types including neurons, and higher resolution microscopy. DISC1/RAB5 co-localisation is little changed following incubation at 37 °C, likely indicating a consistent level of association of DISC1 with early endosomes. The 607F variant has little effect upon DISC1 co-localisation with RAB5, suggesting it does not affect the association of DISC1 with early endosomes. Further to this, analysis of co-localisation between RAB7 and DISC1 showed a moderate association of DISC1 with late endosomes, which is also unchanged after incubation at 37^{O}C. With DISC1-607F there is similar moderate co-localisation between the two proteins under control conditions and after incubation at 37^{O}C. DISC1 may therefore either be a stable component of endocytic vesicles/early endosomes and late endosomes and directly involved in the endocytic pathway, perhaps via regulation of membrane trafficking, or it may be associated with internalised species that pass through the early and late endosomes, or both. Either way, the association of DISC1 with endosomes is apparently not affected by the 607F variant.

DISC1/RAB7 interaction was previously studied by Lepagnol-Bastel et al but the group found no co-localisation between the two proteins, which is at odds with the data presented here (Lepagnol-Bestel et al., 2013). There may be several reasons

for this, first of all the studies performed by Lepagnol-Bastel et al used a DISC1 antibody characterised in an earlier study (Koike et al., 2006) which was shown by western blotting to be specific for the long isoform of DISC1 (~100KD). The specificity of the antibody for immunofluorescent detection of DISC1 has, however, not been demonstrated, and indeed on western blots the antibody nonspecifically detects other proteins, suggesting that any immunofluorescence data generated using this antibody should be interpreted with caution. Moreover, I detected co-localisation of only a small proportion of DISC1 with RAB7-positive endosomes, thus the association may be difficult to detect unless specifically examined at high magnification, which was not the case in the Lepagnol-Bastel study.

Because DISC1 co-localises with endocytic vesicles/early endosomes and late endosomes I determined whether its interactor NDE1 also co-localises with these vesicles. All forms of NDE1 were found to display moderate co-localisation with RAB5 under control conditions. NDE1, NDE1-131A and NDE1-131E were all found to also exhibit moderate co-localisation with RAB7. Like its interactor DISC1, NDE1 therefore co-localises with early and late endosomes. Following incubation at 37^{o}C to allow endocytosis to occur, all forms of NDE1 exhibit reduced association with RAB5. However the association of NDE1 with late endosomes is not substantially altered by internalisation. Like DISC1, NDE1 may therefore be a stable component of late endosomes, but it's association with endocytic vesicles/early endosomes appears to be dynamically regulated. This association with the endocytic machinery is unaffected by the T131 phosphorylation site.

I have shown that DISC1 and NDE1 are within close proximity to components of the NMDA recycling pathway so could potentially modulate NMDA receptor cell surface expression via regulation of endocytosis. At present nothing is known about an endocytic role for DISC1, and little is known about a role for NDE1 in endocytosis, although one study identified a potential role. This study reported that NDE1 is essential for dynein binding to membranes, consistent with its role in regulating dynein activity via LIS1 (Lam et al., 2009). The study also showed NDE1 to be

essential for the correct positioning of organelles such as early endosomes (Lam et al., 2009). Altogether then, NDE1 may recruit active dynein molecules to endosomes for their correct/efficient retrograde transit along the endocytic pathway. Although the T131 phosphosite apparently does not regulate NDE1 association with endosomes, it may, for example, affect its ability to activate dynein, although I detected no effect of the T131 site upon receptor transit along the endocytic pathway. The role of DISC1 may be modulation of trafficking, as has already been observed for synaptic vesicles and mitochondria (Atkin et al., 2010, Ogawa et al., 2013, Mead et al., 2010).

In addition to these published observations I noted a weak effect of NDE1 upon NMDA receptor association with early endosomes under basal conditions that suggests NDE1 may promote receptor association with the endocytic machinery prior to internalisation, but this effect was not robust. Consistent with this I detected dissociation of NDE1 from NMDA receptor complexes following internalisation, which again suggests a role in the early stages of the internalisation process. I did not, however, observe any effect of the NDE1 phosphosite mutants, despite my previous finding in chapter 5 that the NDE1-131E phosphomimic decreases overall NMDA receptor surface expression in comparison to the NDE1-131A phosphonull mutant. The NDE1-131E mutant therefore likely exerts its effects at a different stage of the NMDA receptor trafficking pathway to the endocytic stages examined here.

On the other hand, I did find effects of the DISC1 607F variant, but not of DISC1 itself, upon NMDA receptor internalisation, which is somewhat consistent with the data obtained in chapter 5; In the presence of DISC1-607F I observed no increase in NMDA receptor/RAB5 co-localisation following internalisation, which should normally happen. Thus the variant form of DISC1 either slows down receptor internalisation, or alternatively, speeds it up so that at the time point measured there is no apparent abnormality, although the lack of effect upon NMDA receptor/RAB7 co-localisation suggests that the former is more likely. The results

from chapter 5 show that the 607F variant causes a decrease in surface-expressed NMDA receptors when compared to DISC1 (Figure 5.3). This was thought to be most likely due to either decreased receptor forward trafficking to the cell surface, or to increased internalisation. However my data from chapter 6 suggest that NMDA receptor internalisation is in fact retarded in the presence of DISC1-607F. It is therefore possible that DISC1-607F acts at more than one step in the NMDA receptor trafficking pathway; perhaps inhibiting both forward trafficking and internalisation. Consistent with this speculation, from separate work we now know that the 607F variant reduces net anterograde NMDA receptor movement in neurons (J.K. Millar and E. Malavasi, personal communication). Therefore by inference, DISC1-607F may inhibit transit of NMDA receptors to the cell surface in COS7 cells as well.

I also noted that, unlike NDE1, DISC1 apparently remains stably associated with NMDA receptors during internalisation. It will therefore be interesting to discover what role DISC1 is carrying out. My studies have unfortunately shed little mechanistic light on this, but by showing that DISC1 may affect the rate of forward trafficking and of endocytosis, I have paved the way for such studies in the future.

Next, it would be interesting to determine the effect of the DISC1 species upon surface-labelled NMDA receptor co-localisation with RAB11, a marker of recycling endosomes, and determine if they influence receptor re-insertion into the surface membrane. It would also be interesting to determine if there is any effect of TRAK1 on NMDA receptors endocytosis; this was not studied here due to the overexpression of TRAK1 causing NMDA receptors to aggregate at the mitochondria. TRAK1 knock down and subsequent co-localisation analysis of surface NMDA/RAB5 or RAB11 would one possible way to explore its role.

To follow up the observations made in this chapter it is important to determine if DISC1 or NDE1 co-localise with RAB5 and RAB7 in neurons and if they have similar effects on co-localisation with these proteins after incubation in neurons. Furthermore it would be interesting to determine the effect on receptor membrane

insertion in neurons. A paper by Tang et al devised a novel way to label both recycled and newly inserted NMDA receptors at the surface after incubation. The authors labelled surface receptors at room temperature, and incubated the neurons at 37 °C to internalise the receptors. Excess surface labelled receptors were blocked and the cells were incubated again at 37 °C to allow for recycling of receptors. Cells were then fixed and the surface receptors were labelled with a secondary antibody. Finally the cells were permeabilised and incubated with a secondary antibody to lable receptors which hadn't recycled back to the surface. The co-localisation between DISC1, NDE1 or TRAK1, and recycled or newly inserted receptors could be determined and therefore shed light on any potential role DISC1, NDE1 or TRAK1 might have in the re-insertion of NDMA receptors into the surface membrane (Tang et al., 2010). As discussed in 5.4 the image quality could have been improved by acquiring super-resolution images. These images have a much finer resolution and can take much more detailed images so any co-localisation which occurs could be assessed in a much greater detail.

While the data in this chapter do not provide a mechanistic explanation of the effects of DISC1-607F or NDE1-131E upon surface expression of NMDA receptors in COS7 cells, I have made the novel discovery that DISC1 and NDE1 associate with early and late endosomes, and demonstrated that DISC1 apparently influences the rate of NMDA receptor endocytosis, at least in COS7 cells.

7 Summary of chapters and conclusion

The biological function of DISC1 is still largely unknown. DISC1 plays a critical role in many different developmental processes; however the exact mechanism of how it exerts its function is still poorly understood in many cases. In this thesis I have provided evidence for DISC1 and its pathway partners NDE1 and TRAK1 having an involvement in the trafficking and surface expression of the NMDA receptor. Furthermore I have also shown that polymorphisms within DISC1 and the phosphorylation status of NDE1 can affect this.

DISC1 is a multifunctional multicompartmentalised protein and is well placed to modulate the trafficking of the NMDA receptor (Figure 7.1-1). However the exact points at which DISC1 may modulate NMDA receptor trafficking need to be elucidated. I have provided some evidence for a potential association between DISC1 and the NMDA receptor within the ER. DISC1 has not been previously shown to be present in the ER but co-transfection with GluN1 and GluN2B subunits and co-staining with an ER marker shows co-localisation between DISC1, the ER and GluN1 and GluN2B subunits. Although this needs to be further verified by other experimental methods DISC1 could be well placed to regulate NMDA receptor release from the ER. Both subunits are needed to form a receptor and it is not until the NMDA receptor is fully formed that it is released from the ER (Standley et al., 2000, Scott et al., 2001, Xia et al., 2001). Retention signals and phosphosites regulate the release of the GluN1 subunit from the ER. DISC1 is known to regulate the activity of PKA via its interaction with PDE4 and regulation of cAMP, and specific PKA and PKC sites on the C-terminal tail of the GluN1 subunit are thought to contribute to ER retention/release. Therefore if DISC1 is present within the ER it may play a role in the regulation of the release of the NMDA receptor from the ER via regulation of receptor phosphorylation.

I provided evidence for the 607F variant of DISC1 reducing the expression of surface expressed NMDA receptors in COS-7 cells (Figure 7.1-2). Although I did not find any evidence for wild-type DISC1 modulating the surface expression of the NMDA receptor it is reasonable to think DISC1 does plays a role in the expression of the

NDMA receptor and that DISC1-607F disrupts this. DISC1 is known to link to dynein (Kamiya et al., 2005) and kinesin (Taya et al., 2007) to regulate their transport within the cell and is therefore well placed to modulate the trafficking of the NMDA receptor. Indeed the lab already has data indicating a role for DISC1 in regulating neuronal NMDA receptor trafficking, a role that is compromised by the 607F variant (E. Malavasi & K. Millar, unpublished). Based on all these observations it would be interesting to determine if DISC1 has an effect on NMDA receptor surface expression in neurons.

Finally I showed evidence for the modulation of the internalisation of NMDA receptors (Figure 7.1-3). When co-expressed with DISC1 the levels of internalised NMDA receptor is reduced, indicating DISC1 may play a role in the internalisation of the receptor. I also provided evidence for an association of DISC1 and RAB5- and RAB7-positive endocytic vesicles. This needs to be investigated in more detail but potentially opens new areas for DISC1 research; not only could DISC1 be involved in the trafficking to the surface of the cell it could also be involved in the recycling and/or degradation of the receptor, potentially influencing the trafficking of the NMDA receptor along several points of the pathway from ER release to degradation.

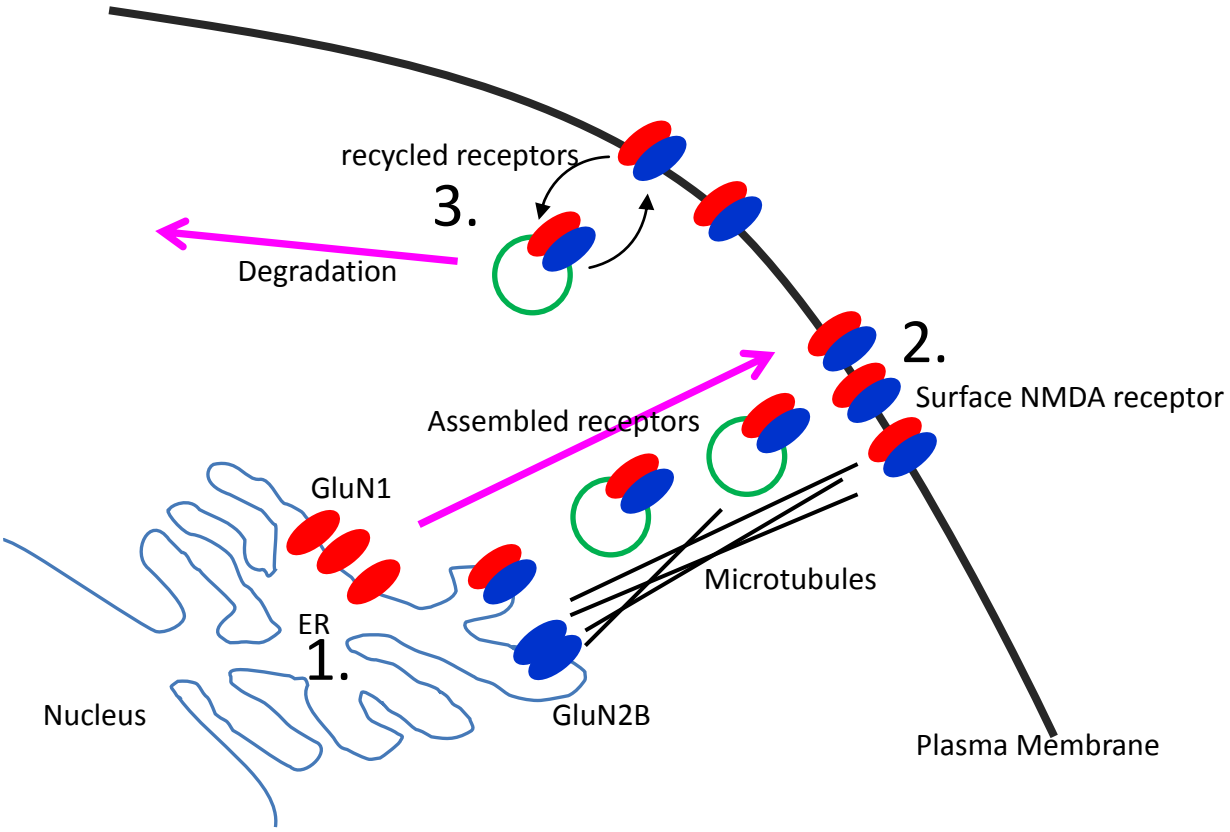

Figure 7.1 The cycle of the NMDA receptor and potential sites of modulation by DISC1

1. NMDA receptors are assembled within the ER from individual subunitsDISC1 may be located near the ER and could affect NDMA receptor release via its regulation of PKA via cAMP through its interaction with PDE4.

2. Assembled receptors are trafficked to the surface of the cell. DISC1 is a known trafficking molecule and, via its association with TRAK1 and NDE1, could be involved in the trafficking of the NMDA receptor. Results presented in this thesis show that the common DISC1 variant 607F can modulate the surface expression of the NMDA receptor Furthermore NDE1 and TRAK1 may also be involved in this process as NDE1 also modulates the surface expression of NMDA receptors, while TRAK1 is robustly associated with the GluN2B subunit.

3. Receptors are internalised and either recycled back to the surface, or removed and degraded. DISC1 and NDE1 both may associate (directly or indirectly) with early and late endocytic vesicles and may therefore be involved in the process of receptor endocytosis and subsequent steps. The DISC1 607-F variant appears to inhibit NMDA receptor endocytosis, but I found no effect of NDE1 upon this process.

NDE1 is a known trafficking molecule critical for the correct positioning of organelles, membrane transport and cell signalling. I showed that NDE1 can modulate the surface expression of the NMDA receptor and the phosphorylation status of T131 may play an important role in regulating this (chapter 4). I also studied whether NDE1 may affect the internalisation of the NMDA receptor but it was found to have no effect. NDE1 did however dissociate from the RAB5 following incubation which, as discussed in chapter 6 could link NDE1 to endocytosis, and via its interaction with dynein could play a role in receptor degradation by trafficking the receptor away from the surface. NDE1 CNVs have been linked to mental illness as well as other disorders of the brain (Hennah et al., 2007, Tomppo et al., 2009). These copy number variants could lead to alterations in NDE1 structure, function or activity. As results in chapters 5 and 6 have shown phosphorylation of NDE1 can affect its function therefore more work is needed to determine if NDE1 may be involved in NMDA trafficking, Knockdown of NDE1 and observing any effect on NMDA receptor trafficking could provide insight into any role NDE1 may have.

Finally I studied the effect of TRAK1 upon NMDA receptor surface expression. I determined that TRAK1 robustly co-localises with the GluN2B subunit of the NDMA receptor which was confirmed by additional experiments performed in the Millar lab. Further to this TRAK1 decreased the level of surface expressed NDMA receptors when co-expressed in COS-7 cells. As discussed in chapter 5 this was largely due to the sequestering of the NMDA receptor at the mitochondria where TRAK1 is known to accumulate when overexpressed. TRAK1 is known to be involved in endocytosis and interacts with EEA1 (Webber et al., 2008) and therefore may also be involved in the recycling of NMDA receptors from the surface of the cell. This could not be studied in the assays performed in chapter 6 due to the sequestering of the NDMA receptor but KD studies could be one way of studying the role of TRAK1 in NMDA receptor endocytosis. TRAK1 specifically co-localises with the GluN2B subunit of the NMDA receptor, and coupled with its known role in trafficking and in endocytosis it is well placed to play a role in the modulation of NMDA receptor trafficking. Knockdown studies of TRAK1 would be the next logical step to study as

overexpression of TRAK1 leads to the mitochondrial clustering of TRAK1 (Brickley et al., 2005) thereby making it difficult to assess any novel effect of TRAK1. It would be interesting to determine if TRAK1 also co-localises with the GluN2A subunit of the NMDA receptor, GluN2B containing NDMA receptors are predominantly expressed in the developing brain before an upregulation of GluN2A containing NMDA receptors in adulthood. If it is a subunit specific interaction this could open up a novel area of research of TRAK1 and NMDA receptors in the developing brain and coupled with the interaction between DISC1, GluN1 and TRAK1 could thereby implicate DISC1. However further research is required to uncover any role TRAK1 may play in NMDA receptor trafficking.

Based on the data presented here DISC1 may form a complex with TRAK1, NDE1 and the NMDA receptor (Figure 7.2). DISC1 is known to associate with both NDE1 and TRAK1 (Millar et al., 2003, Brandon et al., 2004, Burdick et al., 2008, Bradshaw et al., 2008, Bradshaw et al., 2009, Ogawa et al., 2013) and unpublished data shows an interaction between GluN1 and DISC1 (S.Mackie and K.Millar unpublished). Furthermore I have provided evidence for (and further experiments from the Millar lab have confirmed) an association between TRAK1 and the GluN2B subunit of the NDMA receptor, providing further evidence for the formation of a complex. Finally I showed evidence of a potential association between the NMDA receptor and NDE1, although more experiments are needed to confirm this. DISC1, NDE1 and TRAK1 are all known trafficking molecules. Via their association with dynein and kinesin they can modulate intracellular trafficking and organelle positioning. With their individual associations with subunits of the NMDA receptor and their known associations with each other there is evidence for the formation of a DISC1/NDE1/TRAK1/NMDA receptor complex which may regulate the forward trafficking of the NMDA receptor.

Looking at the wider area of DISC1 research and the implication of the data presented in this thesis. The DISC1 family and carriers of the t(1;11) translocation are predicted to produce a C-terminally truncated DISC1 protein (DISC1 1-597). This

predicted protein is truncated before the L607 which has been the focus of study in this chapter, it is reasonable to think that a truncated protein may result in similar decreases in surface NDMA receptor expression as observed in COS7 cells co-expressing L607F-DISC1. Although not replicated by the patch clamping experiments performed in 5.3.2 an effect on NMDA receptor endocytosis was observed in both DISC1 and 607F-DISC1 expressing cells which indicates DISC1 may still play a role in NMDA receptor trafficking via regulation of endocytosis. Disruptions to NMDA trafficking could lead to lower expression of the NDMA receptor which may result in hypofunction of NMDA receptors which is a major theory of mental illness as NMDA antagonists modelled both the positive and negative symptoms of schizophrenia and bipolar disorder (Moghaddam and Krystal, 2012, Krystal et al., 1994). Disruption to DISC1 could also result in alterations in neuronal signalling as the NDMA receptor is the major excitatory receptor in the mammalian brain and is thought to be responsible for memory formation although this is requires further investigation.

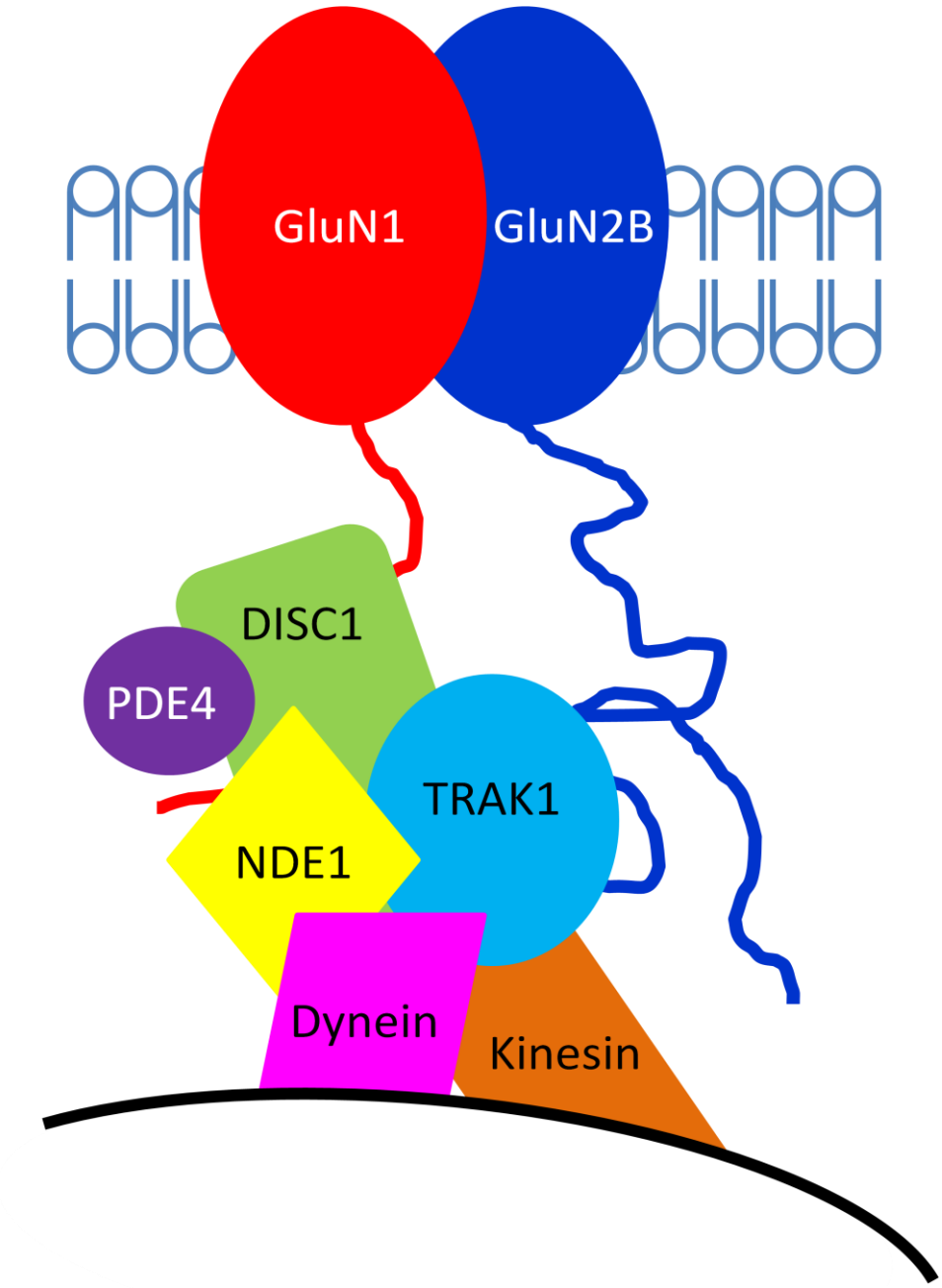

Figure 7.2 Potential complex formation between DISC1, TRAK1, NDE1 and the NMDA receptor

DISC1 has previously been shown to interact with TRAK1 and NDE1 and the NMDA receptor. TRAK1 and DISC1 are known interactors as are kinesin and DISC1, NDE1 and dynein and TRAK1 and dynein. DISC1 and TRAK1 have also been shown to bind to the NMDA receptor and therefore could form a complex which affects the trafficking and surface expression of the NMDA receptor. Adapted from (Millar, 2012).

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